DOCTORAL THESIS

Assessment of Immune Cells Number and Function, Haemorheology and Gene Expression Following Short-term Administration of Recombinant Human Growth Hormone to Healthy Males

Ramos, Sandra

Award date:
2012

Assessment of immune cells number and function, haemorheology and gene expression following short-term administration of recombinant human growth hormone to healthy males.

A thesis submitted for the degree of Doctor of Philosophy

2012

By Sandra Ramos

Faculty of Health Sciences and Medicine

Bond University

Robina - Australia

Supervisors: Associate Professor Sonya Marshall-Gradisnik; Dr. Bon Gray; Professor Mieke Van Driel

Declaration

Submitted in total fulfilment of the requirements of the degree of Doctor of Philosophy by Publication. This thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions. I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text. The work was done under the guidance of Associate Professor Sonya Marshall-Gradisnik, Dr. Bon Gray and Professor Mieke Van Driel at Bond University, Gold Coast - Australia.

_____

Sandra Ramos

Table of contents

3.0 Research studies of this thesis

Acknowledgements

I thank my supervisor Dr. Sonya Marshall-Gradsnik for supporting me and my son along the PhD. I am deeply grateful to Professor Greg Gass who was the person responsible for this great opportunity of doing a PhD at Bond University. Thank you for being an example of professionalism with unique excellence and humbleness. Thank you to Patricia Green (Trish) for supporting and believing in me.

I would like to express my deep and sincere gratitude to Assistant Professor Lotti Tajouri, his wide knowledge and his logical way of thinking have been of great value for me. You were also a good friend along the way.

Thanks for my supervisors Dr. Bon Gray and Professor Mieke Van Driel for so many correction feedbacks.

I wish to express my warm and sincere thanks to Professor Francisco Radler, an idol who was always encouraging and giving personal guidance. He has provided a good basis for the present thesis.

Many thanks to Janet Price for been a great professional and done a fantastic work while working at Bond University supporting any and every postgraduation student.

Special thanks for my friends Siri Lauluten, Adam Sleazak and Ekua Brenu, who supported every decision and fall along the years. Without their constant help for literally everything this thesis wouldn't be possible.

Many thanks for the friendly support of all the crew of PhD students, Rhys Christy, Christian Moro, Mark Barak, Rita Juneji, and my adorable flatmate James Keane.

Many thanks to my loving husband Justin Davey who completes me and makes my life more colourful. Your constant beliefe in me helped me more then you can think.

I owe my loving thanks to my parents Maria Cristina Bahia Ramos and Mario Ramos for their loving support and care. Without their encouragement and understanding it would have been impossible for me to finish this work. Thanks for all the time you both spent on raising Ben with so much love.

My special gratitude is to my precious son Ben who has lost a lot due to my research time demand. You are my biggest motivation of them all!

Papers generated from this thesis

S.B. Ramos, J. F. Brun, B. Gray, S Rogerson, R. Weatherby, L. Tajouri, S.M. Marshall-Gradisnik (2011) The effects of short-term recombinant human growth hormone (rhGH) on blood rheology in healthy young males. *Clin Hemorheol Micro* 47: 121-129.

S.B. Ramos, E. Brenu, R Christy, L. Tajouri, L. McNaughton, B. Gray, M. Van Driel , S.M. Marshall-Gradisnik (2011) Assessment of immune function after short-term administration of recombinant human growth hormone in healthy young males. *Eur J Appl Physiol* 111:1307-1312.

S. Ramos, E. Brenu, F.R.A, Neto, B. Gray, M., Van Driel, L. McNaughton, L. Tajouri, S. Marshall-Gradisnik (2011) Assessment of the gene expression in healthy young males after administration of short-term recombinant human growth hormone. Accepted for publication at the Doping Journal 03/2012.

Refereed Conference Abstracts

S. Ramos, E. Brenu, R. Christy, S. Rogerson, R. Weatherby, L. Trajouri, B. Gray, S. Marshall-Gradisnik (2009) Short-term recombinant human growth hormone and blood rheology in healthy young males. Journal of Science and Medicine in Sport - Australian Conference of Science and Medicine in Sport – Brisbane. Vol. 12, Supplement 2, January 2010, Page e124.

S. Ramos, E. Brenu, R. Christy, S. Rogerson, R. Weatherby, L. Trajouri, B. Gray, S. Marshall-Gradisnik (2009) Immunological effects of short-term recombinant human growth hormone in healthy young males Journal of Science and Medicine in Sport - Australian Conference of Science and Medicine in Sport – Brisbane. Volume 12, Supplement 2, January 2010, Pages e124-e125.

S. Ramos, E. Brenu, R. Christy, S. Rogerson, L. Trajouri, B. Gray, S. Marshall-Gradisnik in Clinical Haemorheology and Microcirculation (2009) Relationship of recombinant growth hormone and blood rheology in healthy young males - 15th Conference for European Society for Clinical Haemorheology and Microcirculation – Switzerland. Vol. 42, Number 3, p.213.

S. Ramos, E. Brenu, L. Trajouri, B. Gray, M. Van Driel, S. Marshall-Gradisnik (2010) Short-term exogenous recombinant human Growth Hormone and its effects on immunoregulatory gene expression. Australian Conference of Science and Medicine in Sport – Port Douglas Vol. 13, Issue 6, p.27.

S.B. Ramos, E. Brenu, L. Tajouri, L. McNaughton, B. Gray, S.M. Marshall-Gradisnik (2011) Short-term Exogenous Recombinant Human Growth Hormone And Its Effects On Immunoregulatory Gene Expression. Medicine and Science in Sports and Exercise, Volume 43, Supplement. 5

List of Tables

Study One

Study Two

Study Three

List of Figures

Study One

Study Two

Study Three

List of Abbreviations

^{125}I – Iodine 125

7AAD - 7-Amino-actinomycin D

ADCC - Antibody-Dependent Cell-Mediated Cytotoxicity

BMD – Bone Mineral Density

°C – Celsius

C9 – Complement Component 9

CD4 – Cluster of Differentiation 4

CD8 – Cluster of Differentiation 8

cDNA – Complementary Deoxyribonucleic Acid

CO_2 – Carbon Dioxide

CT – Threshold cycle

DCM – Dilated Cardiomyopathy

DNA – Deoxyribonucleic Acid

EA – Erythrocytes Aggregation

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

ERKs - Extracellular Regulated Kinases

FDA – Food and Drug Administration

Fig - Figure

GH – Growth Hormone

GHBP – Growth Hormone Biding Protein

GHD – Growth Hormone Deficiency

GHR – Growth Hormone Receptors

GZMA – Granzyme A

h - Hours

HCT - Hematocrit

HIV – Human Immunodeficiency Virus

IFN-γ – Interferon Gama

IGFBPs - Insulin Growth Factor Binding Proteins

IGF-I – Insulin Growth Factor – I

IGF-II – Insulin Growth Factor – II

IGF-R – Insulin Growth Factor Receptor

IL10R – Interleukin 10 receptor

IL2 – Interleukin 2

IL4 – Interleukin 4

IL6 – Interleukin 6

IL6R – Interleukin 6 receptor

ImA – Immunoglobulin A

ImG – Immunoglobulin G

ImM – Immunoglobulin M

IU – International Units

JAK – Janus Kinase

K562 - Human erythromyeloblastoid leukemia cell line

Kg – Kilogram

LBM – Lean Body Mass

m - Meters

M1 – Mode One

M2 – Mode Two

MAPK– Mitogen-Activated Protein Kinase

MCP-1 - Macrophage Chemoattractant Protein-1

MF(AU) – Mean Fluorescence Intensity (Arbitrary Units)

mL - Millilitres

mRNA – Messenger Ribonucleic Acid

n – Number

N – Newton

NCBI – National Center for Biotechnology Information

NFκB – Nuclear Factor Kappa B

NK – Natural Killer Cell

NKCA – Natural Killer Cell Activity

N.m – Newton meters

P – Placebo

PBMC – Peripheral Blood Mononuclear Cell

PBS – Phosphate-buffered saline

PCR – Polymerase Chain Reaction

PFR1 – Perforin 1

PKH26 - Red Fluorescent Cell Linker

Q-RTPCR – Quantitative Reverse Transcription PCR

RBC – Red Blood Cell

RCF – Relative Centrifugal Force

rhGH – Recombinant Human Growth Hormone

RIA – Radio Immuno Assay

RNA – Ribonucleic Acid

RT-PCR – Real Time Polymerase Chain Reaction

SPSS - Statistical Package for the Social Sciences

STAT – signal transducer and activator of transcription

Th – T Helper Cell

Th1 – T Helper Cell Subset One

Th2 – T Helper Cell Subset Two

TS – Turner Syndrom

WADA – World Anti-Doping Agency

WBC – White Blood Cell

Abstract

Introduction: Recombinant human growth hormone (rhGH) has been extensively used by healthy young males and athletes in the attempt to improve body mass and sports performance. The expression of GH receptors (GHR) has been reported previously by dual fluorochrome flow cytometry on human peripheral blood lymphocytes (PBL). In the human, rat, and dog immune systems the presence of GH and GH mRNA, as well as their cellular distribution in the spleen, thymus, bone marrow, tonsils, lymph nodes, liver and in leucocytes has been reported in the literature. Although many studies have addressed the ability of the immune system to control pituitary GH secretion, no studies have been reported on the effect of the immune activation due to rhGH in healthy population. The *in vitro* growth-promoting effect of hGH upon human erythroid precursors was previously proven to be mediated by paracrine IGF-I. However, the effects of rhGH on the blood rheology of healthy population are still to be evaluated. Hence this PhD is composed of three main investigations. Study One explored the effects on the blood rheology (red blood cell aggregation, deformability and plasma fibrinogen) parameters in healthy young males due to short-term administration of rhGH. These parameters can be used to estimate the efficiency of the O_2 supply for working tissues and perhaps may indicate possible effects of athletes' performance. Study Two examined the immunoregulatoty effects on: NK cell number, activity and phenotype, T cell subsets, $CD4^+/CD8^+$ ratio, $CD4^+$ (Th1/Th2) cytokine production of Interleukin 2 (IL2), Interleukin 4 (IL4), Interleukin 6 (IL6), Interleukin 10 (IL10), Tumor Necrosis Factor - alpha (TNF-α) and Interferon – gamma (IFN-γ) after short-term administration of rhGH in healthy young males. The immunoregulatory parameters were investigated because they provide an indication of the health status of the subjects. Study Three examined the regulation of gene expression in selected immune genes: Interleukin 6 (*IL6)*, Interleukin 6 receptor (*IL6R)*, Interleukin 10 receptor (*IL10R)*, Perforin (*PFR1)*, nuclear factor kappa B (*NFKB)* and

Granzyme A (*GZMA*). The evaluated genes are representative of immune pathways potentially affected by the study intervention. The correlation between these immune and rheologic parameters and IGF-I production were also investigated. **Methodology:** In Study One 30 males (27 ± 9 yrs) were randomly assigned to either placebo (n = 15) or rhGH (drug group) (n = 15). The drug group was given daily injections of 1mg/day of rhGH for seven days. Myrenne aggregometer was used for red blood cell (RBC) aggregation indexes. Deformability was evaluated by Ektacytometer and the elongation index was calculated from the light intensity along the major and minor axes of the diffraction image. Plasma fibrinogen concentration was determined quantitatively using the Von Clauss clotting method. In Study Two, 30 males (27 ± 9 yrs) were randomly assigned to either placebo (n = 15) or rhGH (drug group) (n = 15). The rhGH group was given daily injections of 1mg/day of rhGH for seven days. Peripheral blood mononuclear cells (PBMC) were assessed via flow cytometry and data was generated at baseline and Days 8, 15, 22 and 29 post injection for: NK cell number, activity and phenotype, T cell number, $CD4^+$ (Th1/Th2) cytokine production of IL2, IL4, IL6, IL10, TNF-α and IFN-γ and $CD4^+/CD8^+$ ratio with particular attention to the possible correlation to IGF-I production. In Study Three, 30 males (27 ± 9 yrs) were randomly assigned to either placebo (n = 15) or rhGH (drug group) (n = 15). The drug group was given daily injections of 1mg of rhGH for seven days. The gene expression in PBMC was assessed by microarray and confirmed by RT-PCR for the rhGH and placebo groups. Gene expression data was generated at baseline and Days 8, 15, 22 and 29 post injections. All data were analysed using General Linear Model with repeated measures, Bonferroni correction factor and significance was set at $p \leq 0.05$. **Results:** In Study One there was a significant increase in the erythrocytes aggregation index post injection (day 8), in accordance with an increase in IGF-I levels in serum. In Study Two a significant time effect was noted in IL10 secretion (pg/mL) from Day 15 (P= 35.14 ± 19.93, rhGH = 26.63 ± 16.39) to Days 22 (P= 61.32 ±

20.41, rhGH= 74.99 ± 46.91) and 29 (P= 101.98 ± 67.25, rhGH= 107.74; ± 122.58). There was no correlation between IGF-I levels and immune cells numbers or activity. In Study Three the ratio of gene expression at each time point post baseline (Days 8, 15, 22 and 29) was normalised against baseline differential expression. On day 8 all the evaluated genes presented an up-regulation expressed as the ratio against baseline in the rhGH group compared with the placebo group. Interestingly, from Day 8 to Day 29 all the evaluated genes except *IL6* remained up regulated in relation to baseline in the rhGH group. Serum IGF-I levels (ng/mL) increased significantly ($p \leq 0.01$) on Day 8 (0.48 ± 0.78) after injections compared to baseline (0.31 ± 0.07) and Days 15 (0.33 ± 0.06), 22 (0.29 ± 0.05) and 29 (0.29 ± 0.06). **Conclusion:** This thesis contributed to the body of the literature by generating unique data on the use of rhGH by healthy young subjects but mostly it provided data to educate and inform the effects of the use of rhGH by athletes, coaches or healthy young people with normal levels of GH. The results of the three studies performed have shown that short-term administration of 1mg/day of rhGH in healthy young males meet the three main criteria for the inclusion of GH on the list of prohibited substances once it was concluded that 1mg/day of rhGH leads to acute adverse effects in the erythrocytes aggregation through the increase in the native blood aggregation index. Moreover, our results potentially suggest a chronic effect of rhGH on specific anti-inflammatory cytokine (IL10) release suggesting a possible advantage for the immune function of athletes and healthy young males with the administration of rhGH and consequently an unfair advantage in the sport performance and demonstrated a novel and interesting pattern of anti-inflammatory profile following rhGH administration. Such advantage was also shown in Study Three as rhGH was effective in up-regulating the anti inflammatory *IL10* gene pathway through the up-regulation of *IL10R* confirming the possible advantage in the performance of athletes after administration of

rhGH. Importantly, this thesis has shown the possibility of using genome evaluation to assess new pathways for detection of rhGH in healthy male athletes.

Chapter One

Introduction

1.0 Introduction

According to the World Anti-Doping Agency (WADA, 2009), doping can be defined as the presence of a prohibited substance, or its metabolites or markers, in an athlete's bodily specimen. (WADA, 2009). Doping in sport has a very long history beginning in the original Olympic Games in 1896 (Sonksen, 2001).

Many factors contribute to both top level and amateur athletes' use of banned substances, such as the prospect of enormous earnings, the desire to become famous and raising an individual's social status (Aquino Neto, 2001). However, the biological effects of substances such as growth hormone (GH) and its ability to improve sports performance in healthy population are based on anecdotal evidence.

In clinical studies, recombinant GH is used to treat severe GH deficiency both in children and adolescents, such as idiopathic short stature (Bryant, et al., 2007), chronic renal insufficiency (Haffner, 2000), in short prepubertal children with nephropathic cystinosis to improve height velocity and stature height (Wuhl et al., 2001). It is also applied with significant benefits in children born smaller than their required gestational age, AIDS-associated wasting and mal-absorption associated with short bowel syndrome (Napolitano et al., 2008). In patients with heart failure treated for 3 months with GH (4 IU every other day), GH induced a growth response in the diseased myocardium and reshaped left ventricular geometry with a marked fall in systolic wall stress. The ejection phase indices, ventricular mechanics, and cardiac performance improved considerably (Fazio et al., 1996).

Johannsson and colleagues (1996) applied daily doses of recombinant GH for two years in patients with adult-onset GH deficiency (24 men and 20 women; aged 23-66 yr). The initial dose was of 4.8 μg/kg and target dose of 12μg/kg. The doses were reduced due to side effects

(not specified by authors) associated with taking a maximum dose of 7.8µg/kg. Bone mineral density (BMD) and bone metabolism were assessed with dual energy x-ray absorptiometry, and serum concentrations of osteocalcin, carboxy-terminal propeptide of type I procollagen, and carboxy-terminal cross-linked telopeptide of type I collagen. Two years after treatment there was a significant increase ($p<0.05$) in the lumbar spine L2-L4 by 3.8%, in the femoral neck by 4.1%, trochanter by 5.6%, and in Ward's triangle by 4.9%. Although the dose had to be adapted later in the study, the results due to the treatment suggest a positive effect of the recombinant GH in the BMD and bone metabolism in the GH deficient population. Similarly, in an elderly population consisting of 12 males and 4 females aged 62-74 yrs, a single subcutaneous dose of GH (0.03mg/kg) 3 times per week in a 3 months trial revealed a significant increase in lean body mass ($+3.3 \pm 0.7$kg, $p \leq 0.01$) for the GH group as well as a significant increases in mean muscle mass ($+3.3 \pm 1.1$kg, $p < 0.02$) (Welle et al. 1996). Furthermore, GH therapy has been applied in girls with Turner's syndrome. The outcomes of the therapy resulted in a final height of 150.4± 5.5 cm, which was 8.4±4.5 cm more than the predicted height in the absence of therapy (Rosenfeld et al., 1998).

Although studies have indicated significant positive outcomes such as increased lean body mass (LBM), bone mineral density (BMD) (Johannsson et al., 1996), improved cardiac performance (Fazio et al., 1996) in specific population groups, those desired performance enhancement effects of GH in athletes or healthy populations have not been successfully examined. The paucity of studies with healthy subjects may be due to limited availability of subjects to be admitted into these kind of studies, the ethical application of GH in healthy people, the appropriate dosage to be administering that would accurately reflect the athletes' degree of misuse of GH and also the likelihood that GH may have been administrated per athletes concurrently with other anabolic substances which may mask the individual effects of GH.

There is evidence that the misuse of GH causes side effects such as: sodium and water retention (acute onset), diabetes, cardiovascular diseases, hypertension, cardiac deficiency, acromegaly and accelerated osteoarthritis (delayed onset) (Wallace et al., 1999, Melmed, 2006). In addition previous researchers have identified GH receptors present on specific immune cells, such as $CD20^+$ cells (B cells), were expressed in much lower amounts in $CD2^+$ cells (T cells, NK) (Badolato et al., 1994). Rapaport et al., 1995 showed that GH-R was expressed in more than 90% of B-lymphocytes and monocytes and in 2-20% of T cells using fluorescein isothiocyanate (FITC-GH), suggesting a possible stimulatory effect on cytokine production, cytotoxic activity of NK cells, B cell function and proliferation.

The relationship between GH and immune cells is outlined when mice, which have undergone myeloablative therapy, achieve normal levels of haemoglobin, white blood cells and platelets much more rapidly when given GH (Tian et al., 1998). Importantly, Badolato and co-workers (1994) used a two-colour flow cytometric analysis with a monoclonal antibody specific for GHR (mAb263) and the GH ligand itself, to show the presence of GH receptors in B- lymphocytes, T- lymphocytes and Natural killer (NK) cells in humans. Although the authors had concluded that GH may have important regulatory effects on B-lymphocytes it also exhibited considerably lower levels of receptor expression on T-lymphocytes and NK cells. Similarly, by administering 700 μg of human GH intramuscular for an average of 14 days Crist et al., (1987) evaluated natural killer cytotoxic activity (NKCA) in women with impaired GH secretion and concluded that daily exogenous GH treatment favourably increases NK cell activity.

In agreement with the effects of GH on the immune function, Sitz (1990) after analysing four patients with similar disorders could associate the deficiency of GH with hypogammaglobulinemia (immune disorder due to low levels of immunoglobulin G (IgG),

immunoglobulin A (IgA) and/or immunoglobulin M (IgM)). This suggests a potential effect of GH on the immune system. It is also known that GH primes the oxidative burst of human neutrophils (Fu et al., 1992) and decreased neutrophil numbers can lead to increased rates of infection as a result of a decreased capacity to neutralise immunogens (Wenisch et al., 2000).

It has been suggested by Dimitrov et al., (2004) that hormonal changes characterised in early nocturnal sleep (such as with GH), would be responsible for a shift towards T helper 1 (Th1) cytokines. Based on their results, the authors suggest that enhanced GH concentrations during early nocturnal sleep synergistically act to enhance Th1 cytokine activity. The question is whether the same effects would be characterized due to recombinant human growth hormone (rhGH) administration in healthy subjects. Further evidence for GH potentially exerting its effects is evident where GH has been shown to induce erythropoieses (Brun, 2002). However, the haemorheology properties of, and the effects on, GH have not been examined. Based upon these findings, the importance of examining the interaction of the administration of GH and the potential effects on the immune function of a healthy population are of significance.

While there are anecdotal affirmations of GH misuse not only by elite athletes but also by recreational athletes, it is imperative to further investigate the effect of short-term administration of GH on blood rheology, immune function and gene expression in healthy active adults. The use of rhGH in the aggregation and deformability of erythrocytes, which impact on blood rheology (Baskurt et al., 2004) and consequently on sports performance needs investigation and assessment.

Peripheral blood mononuclear cells (PBMCs) are reported to express some 75% of the human genome (Gladkevich et al., 2005), and have been shown to express receptors for (and thus potentially transcriptional profiles that are modified by) agents such as GH (Asakawa et al., 1986). A change in gene sequence affects the morphology and phenotypic properties of

proteins (Lockhart & Winzeler 2000) and this regulation of gene expression is a necessary component of optimal eucaryotic function. This can be achieved by adjusting protein activity, generating protein diversity and controlling the concentration of a protein in a particular area (Orphanides & Reinberg 2002). In particular, the assessment of the expression of genes linked to the activity of the immune system may represent the effects of the use of short-term administration of rhGH in healthy young males at the gene level.

Based upon the limited previous investigations examining GH and their potential effect on healthy population the proposed research is of particular significance for providing evidence of the effects of rhGH in the blood rheology (aggregation, deformability), on immune cells and their function, and in the gene expression of specific immune genes of healthy young males.

Chapter 2

Review of the literature

2.1 Growth hormone

Growth hormone (GH, somatotrophin) is a single chain polypeptide containing 191 amino acids, two disulfide bridges and four helical structures (Nussey and Whitehead, 2001). The half life of GH in circulation is approximately 20 minutes (Veldhuis et al., 1993). It circulates in several forms that vary according to size (molecular weights of 20 KDa and 22 KDa), isoelectric point (acidic forms), oligomers (up to pentamers) and fragments (molecular weights of 12 KDa and 16 KDa) (Nussey and Whitehead, 2001). In addition, approximately 50% is bound to the extracellular domain of its receptor (also termed the GH-binding protein, GHBP).

There are two molecular variations of the GH receptor, full-length and truncated form. The full-length form belongs to the Class I cytokine receptor family (Nussey and Whitehead, 2001). These are all proteins of the single transmembrane domain in which the intracellular domain is associated with a protein tyrosine kinase known as Janus kinase (JAK), that phosphorylates signal transducers and activator of transcription (STAT) kinase (the JAK-STAT signalling pathway) initiating a cascade of protein phosphorylation events (Nussey and Whitehead, 2001).

Classically, the synthesis and secretion of GH has been thought to be controlled by two hypothalamic neurohormones, stimulatory growth hormone-releasing hormone (GHRH) and inhibitory somatostatin (inhibits GH's secretion) (Bouillanne et al., 1996). However, Ghrelin is another hormone found to be responsible for the release of GH (Kojima et al., 1999). Ghrelin was shown to function in humans more powerfully as stimulus for GH production than GHRH (Arvat et al., 2000). The mRNA for Ghrelin and its receptor were found in all human tissues including lymph nodes, circulating lymphocytes and spleen (Gnanapavan et

al., 2002). Although in this study the actual class of lymphocytes was not specified, the potential for GH to elicit a response on immune cells is evident.

2.2 GH receptors

Growth hormone receptor (GHR) consists of a single chain of 638 amino acids including an 18 amino acid signalling peptide, a 246 residue extracellular cytoplasmic domain, a small 24 amino acid transmembrane domain and a 350 residue intracellular cytoplasmic domain (Postel-Vinay, 1994). While the liver is one of the most abundant sources of GHR, improved detection techniques combined with analysis of mRNA expression, provide evidence that GHR are present in adipose tissue, heart, kidney, stomach, small intestine, colon, pancreas, lung, brain, cartilage, muscle, adrenal gland, skin, lymphatic cells, testis, ovary, corpus luteum, mammary gland and specifically the immune cells (Mathews et al., 1989; Tiong and Herington, 1989; Lobie et al., 1990; Tiong and Herington, 1992). Studies using Crystallography have demonstrated that the addition of GH to GH binding protein (GHBP) results in a complex in which two GHBP molecules are bound by a single GH molecule (deVos et al., 1992). The binding occurs in a sequential manner where one GHBP molecule binds to site 1 on GH and then the second GH site becomes available for a second GHBP molecule to bind.

2.3 GH regulation

Growth hormone concentrations (22KDa) rise after birth reaching level concentrations higher (4 days old neonates 11.20 ± 1.97 ng/mL) than that of healthy adults (12 men, 8 women), aged 20 ± 0.3 years old (2.43±0.72 ng/mL) (Radetti et al., 2000). The most profound effects are by stimulating proliferation of cartilage in the epiphyseal plates of long bones before they fuse. In addition to stimulating linear growth, GH also increases total bone mass and mineral

content by increasing the activity and number of bone modelling units (Nussey and Whitehead, 2001). A peak period is observed during puberty and there is a marked decline in old age. In the adult human, approximately five pulses of GH are secreted during a 24 h period with a largest peak occurring at the onset of sleep. The mean concentration of circulating GH varies throughout life (Nussey and Whithead, 2001) and in healthy adults GH concentrations are less than 3 ng/mL (Shimizu et al., 2005). In normal subjects (6 men and 6 women, aged 20-47 yr) under physiological conditions, plasma GH, as measured by IRMA, was detectable at all time points during 24h evaluation and ranged from 40 to 19.695 ng/L (Winer et al., 1990).

Growth hormone has direct actions on the liver, adipose tissue and muscle although many of its actions are mediated by increasing the synthesis and release of insulin-like growth factors (IGFs) (Savino et al., 2002). Insulin Growth Factors (IGFs) stimulates DNA, RNA and protein synthesis in many organs such as thymus (Savino et al., 2002), increasing both their size and function including thymocyte proliferation and migration (Savino et al., 2002). The Growth Hormone-stimulated release of IGFs from the liver also has important feedback effects on the control of GH release, inhibiting it. Growth hormone also stimulates the synthesis and release of insulin-like growth factor binding proteins (IGFBPs) which bind circulating IGFs (Guler et al., 1989). This binding provides a reservoir of circulating IGFs. Normal concentrations of GH are also required to sustain normal pancreatic islet function. Thus, during GH deficiency insulin secretion declines whilst an excess of GH reduces insulin-dependent glucose uptake causing a rise in insulin secretion to compensate for the GH-induced resistance (Guler et al., 1989).

2.4 Clinical uses of GH

Growth hormone therapy is the standard of practice by Food and Drug Administration (FDA) approved for children of short stature with classic GH deficiency, chronic renal failure, and Turner's syndrome. The Canadian Growth Hormone Advisory Committee conducted a study in 2005 where 154 girls with Turner Syndrome (TS) (aged 7–13 years) were randomly assigned to receive GH (0.3 mg/kg/week, maximum weekly dose 15 mg) or no treatment until height velocity was less than 2 cm/year and bone age was greater than 14 years. After a mean follow up period of 5.7 ± 1.6 years, the GH group achieved a final adult stature 7.2 cm taller than the control group (The Canadian Group Hormone Advisory Committee, 2005). The results suggest that replacement with GH to GH deficient population has positive outcomes and is standard of practice for treatment.

Adamopoulos et al., (2003) studied the effects of subcutaneous administration of GH (4 IU GH every other day) in 12 idiopathic dilated cardiomyopathy (DCM) patients (50± 4 years) in a 12- week treatment period against a 12-week period without treatment with GH on circulating pro-inflammatory/anti-inflammatory cytokine balance, and whether these GH-induced immunomodulatory effects were associated with the improvement of left ventricular (LV) contractile performance in DCM patients or not. The authors demonstrated, through enzyme-linked immunosorbent assay (ELISA), that a 3-month therapy with GH causes a significant decrease in the circulating proinflammatory cytokines (tumour necrosis factor-α (TNF- α), interleukin-6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF) and its soluble receptor GM-CSFR), chemotactic chemokines, macrophage chemoattractant protein-1 (MCP-1) and soluble adhesion molecules intercellular adhesion molecule-1 (sICAM-1) and vascular cell adhesion molecule-1 (sVCAM-(1)), as well as an increase in the serum anti-inflammatory/pro-inflammatory balance (IL-10/IL-6, IL-10/TNF- α and TGF-

β2/TNF-α) whilst enhancing contractile reserve and reducing LV dimensions. Those results suggest an important role of GH at immunomodulatory effects for cytokines balance.

In another clinical area, Haffner et al., (2000) determined the final adult height of 38 children with chronic renal failure who were treated with GH for up to nearly nine years. The results were compared to 50 similar children who did not receive the GH therapy because their growth retardation at the beginning of the study was less marked than that of the treated children. The mean final adult height of the growth hormone–treated children was 1.6±1.2 SD below normal, which was 1.4 SD above their standardized height at base line ($P< 0.001$). Differently, the final height of the untreated children (2.1±1.2 SD below normal) was 0.6 SD below their standardized height at base line ($P<0.001$).

Similarly, Li and co-workers (2008) evaluated the efficacy and safety of rhGH in the treatment of 114 patients with chronic liver failure, where 56 patients in the rhGH treatment group received 4.5 IU of rhGH intramuscularly daily for 4 weeks while 58 patients were assigned in the control-treatment group and 15 healthy subjects served as controls. The study showed the efficacy of rhGH treatment was 87.5% (vs. 38.1% in the controls-treatment group, $p < 0.01$) and the survival rate of the rhGH treatment and control-treatment groups after 2 weeks, 1 month, 3 months, and 6 months of treatment was 98.21% vs. 75.86%, 91.07% vs. 62.07%, 66.07% vs. 22.41%, and 55.36% vs. 13.79%, respectively. The response from the use of GH in clinical treatments is widely observed as previously shown however to date experiments have not been conducted in healthy populations.

2.5 Doping with GH in sport

Studies examining the potential performance enhancing effect of GH are limited provided that GH is an illegal substance part of the list of prohibited substances by the World Anti-Doping Agency (WADA). Administration of rhGH in a double-blind study (16 males of 21-

34 yr) prior to and during a 12-week programme of daily resistive weight training (75-90% maximum strength with low repetition (4-8 times) for 4 sets/session 5 days/week) while receiving 40 μg rhGH.kg-1.day-1 or placebo resulted in no significant difference between the two groups with regards to muscle strength, size and protein synthesis (Yarasheski et al., 1992). In this study muscle strength was determined via maximum amount of weight lifted on each of the Nautilus exercise devices (N.m). The maximum force produced by the knee extensor and flexor muscles during maximum voluntary concentric (60°/s) and isometric contractions on Cybex dynamometer, while whole protein turnover was measured by [^{15}N]glycine was administered orally (0.5mg ^{15}N kg-1.day) every 3 h during the last 60 h of each 10-day controlled protein diet period. The ^{15}N enrichment in total urinary N was determined, and the rates of whole body protein turnover, synthesis, and breakdown were calculated as previously described (Steffee et al., 1976).

Similarly, no significant difference ($p>0.05$) was found in bone mineral accumulation measured by dual-energy X-ray absorptometry. Yarasheski et al., (1997) evaluated 18 elderly healthy men (67± 1 yr) during 16 weeks of progressive resistance training (75-90% maximum strength, 5-10 repetitions/set, 4 sets/day, 4 days/week). Participants in this study received either daily 12.5 or 18 μg/kg GH, (n=7) or placebo (n=11). The daily dose of GH did not enhance the whole body or regional bone mineral density (Yarasheski et al., 1997). Similarly, in support of these previous studies Yarasheski (1993) examined 7 healthy young (23 ± 2 yr; 86.2 ± 4.6 kg) experienced weightlifters for 14 days with subcutaneous GH administration (40 microgram.kg^{-1} x day^{-1}). There was no increase in the rate of muscle protein synthesis or reduced rate of whole body protein breakdown determined during a constant intravenous infusion of [^{13}C]leucine (Yarasheski et al., 1993).

Based upon these limited findings there seems to be little evidence to suggest increased sport performance after GH administration. However, it is highly plausible that elite athletes may be using GH in dosages ranging from approximately 15 to 180μg/kg per day, which would be higher than dosages used in most experimental studies (Saugy et al., 2006). Furthermore, anecdotal reports of sports doping regimens suggest that GH is not typically used as a single agent, but rather is often combined with other substances, such as anabolic androgenic steroids, insulin, and anti-estrogens (Fainaru-Wada, 2006) suggesting that they may have different benefits and risks when taken together.

2.6 IGF-1

Insuline growth factor-I is a polypeptide promoting growth cellular consider, consequently, essential for the normal development. The two ligands, IGF-I and IGF-II, are mitogenic peptides that are highly homologous to each other and share structural homology with insulin. The insulin-like growth factor system is composed of a family of interacting ligands, receptors and binding proteins (Daughaday et al., 1989). The hepatic synthesis of IGF-I is largely GH dependent, whereas the synthesis of IGF-II is relatively independent of GH. (Khandwala et al., 2000) It was recently shown that IGF-1 is inversely correlated with IL-6 plasma levels (Barbieri et al., 2003). In a longitudinal survey it has recently been shown that older women having low serum levels of IGF-I and high serum levels of IL-6 have the highest risk of disability and mortality, in comparison with women who have low levels of IL-6 and high levels of IGF-1 (Cappola et al., 2003).

2.7 Immune system

Studies confirming GH influences on the immune system, originated prior to 1987 from findings in hypophysectomised animals (rodents) as well as in humans with deficiencies in

pituitary hormones (Gala, 1991). Immune cells are able to produce GH (Varma et al., 1993), IGF-I (Nyman and Pekonen, 1993) and IGF-binding proteins (Nyman and Pekonen, 1993). Growth hormone acts directly on many tissues by binding to the GHR (Smit & Carter-Su 1996).

Both innate immunity and adaptive immune responses depend on the activities of leucocytes (Murphy et al., 2007), cells of the immune system that defend the body against both infectious disease and foreign pathogens. Innate immunity largely involves activities of granulocytes and macrophages (Murphy et al., 2007). Cells of the adaptive immune response include lymphocytes, which have the capacity to promote life-long immunity after exposure to disease or vaccination. The innate and adaptive immune systems together provide a remarkably effective defence system (Murphy et al., 2007). Many infections are successfully eliminated by the innate immune system; others that cannot be resolved by innate immunity trigger adaptive immunity and are then overcome successfully and prevented from recurring by immunological memory (Cooper and Alder, 2006). Several different types of leucocytes exist, all produced and derived from hematopoietic stem cell in the bone marrow (Cooper and Alder, 2006).

The number of leucocytes in the blood is often an indicator of disease. There are normally between 4×10^9 and 11×10^9 white blood cells in a litre of blood, making up approximately 1% of blood in a healthy adult (Alberts, 2005). There are two major types of lymphocyte: B lymphocytes, which when activated differentiate into plasma cells that secrete antibodies; and T lymphocytes.

This project selected the following immune cells to be investigated: NK cell number, activity and phenotype, T cell number, $CD4^+$ (Th1/Th2) cytokine production of IL2, IL4, IL6, IL10, TNF-α and IFN-γ and $CD4^+/CD8^+$ ratio.

2.7.1 T Lymphocytes

The T cells contribute to immune defences in a direct way where it regulates immune responses; or directly attack infected or cancerous cells. In most cases, T lymphocytes only recognize an antigen if it is carried on the surface of a cell by one of the body's own or major histocompatibility complex (MHC), molecules (Murphy et al., 2008). The T lymphocytes population is grouped in two; helper T lymphocytes which mainly express cluster of differentiation four ($CD4^+$) and cytotoxic T lymphocytes which primarily express cluster of differentiation eight ($CD8^+$) surface proteins (Murphy et al., 2008). The $CD4^+$ T lymphocytes have a regulatory overall effect on immunity and orchestrate their activity through diverse cytokine secretions or by direct intercellular contact (Murphy et al., 2008). The $CD8^+$ cytotoxic T lymphocytes are effector cells that secrete and respond to cytokines targeting infected cells to induce programmed cell death (Kimata and Yoshida, 1994). The T cell immunological activity and response can be monitored by assessing the $CD4^+/CD8^+$ ratios that illustrate critical T cell functions (Kimata and Yoshida, 1994). Previous *in vitro* studies have shown that GH effects on normal and neoplastic human T cells enhanced proliferation is mediated by local increase expression of IGF-I (Geffner et al., 1990; Merchav et al., 1988; Mercola et al., 1981; Mosmann and Coffman, 1989).

2.7.2 CD4/CD8 ratio

The CD4/CD8 lymphocyte ratio in blood is used in the diagnosis of HIV infection (Pahwa et al., 2008; Butt et al., 2007; Shearer et al., 2007) and autoimmune disorders such as rheumatoid arthritis or vitiligo (Hussein et al., 2008; Pichler et al., 2009). The ratio of CD4/CD8 is used as a marker of the immune system function (Evans et al., 2004) determining the ratio of the T helper/suppressor profile.

2.7.3 Th1 and Th2

The difference between these two subtypes of lymphocytes pertains to the type of cytokine they produce; Th1 cells secrete interleukin four (IL-4) and Th2 cells secrete interferon-gamma (IFN-γ) (Mosmann, 1996). Naïve T lymphocytes and mature T lymphocytes in general express certain co-stimulatory receptors that are important for fighting against infection (Murphy et al., 2008). Th1 responses include cell mediated reactions that are important for dealing with cellular pathogens, whereas Th2 responses regulate production of antibodies in response to extracellular pathogens and mediate allergic processes (Romagnani, 1999). An example of a co-stimulatory receptor is CD28 which is induced during infections. It is essential for the propagation of T cells and cytokines (Murphy et al., 2008). In the presence of an infection, professional antigen presenting cells (APC) such as dendritic cells or cytokines bind to the CD28 expressed on the surface of the naïve T cells, thus activating the T cell (Murphy et al., 2008).

2.7.4 Natural Killer

A third lineage of lymphoid cells, called natural killer (NK) cells, lack antigen specific receptors and are part of the innate immune system (Perricone et al., 2008). They are able to recognise and kill some abnormal cells, for example some tumour cells and virus-infected cells, and are thought to be important in the innate immune defence against intracellular pathogens (Murphy et al., 2007). There are two main types of NK cells; 90% of these cells are classified as $CD56^{dim}CD16^{+}$ while the remaining 10% are comprised of $CD56^{bright}CD16^{-}$ (Farag et al., 2002). $CD56^{bright}CD16^{-}$ facilitates the production of cytokines while $CD56^{dim}CD16^{+}$ induces both natural cytotoxicity and Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC), hence there are high levels of lytic granules in $CD56^{dim}CD16^{+}$ compared to $CD56^{bright}CD16^{-}$ (Farag et al., 2002).

Firstly described by Canty and Wunderlich (1970), the ^{51}Cr release assay has been criticised for requiring a radioactive isotope and for not permitting the characterization of the cells involved in the cytotoxic reaction (Lecoeur et al., 2001). Additionally the chromium (^{51}Cr) release assay is more expensive and the spontaneity involved in this protocol makes it more disadvantageous (Neri et al., 2001). The proposed method measures the ability of the NK cells to lyse the erythroleukemia cell line known as K562. The principle behind this method is that the NK cells once in contact with the K562 cells cause them to undergo apoptosis by activating cell death pathways. Therefore this method assess the number of K562 that have undergone apoptosis in the presence of NK cells and compares them to a population of live K562 that were not induced to undergo apoptosis with NK cells (Zamai et a., 1998).

2.8 Immune cell function and GH

Hormones are immunomodulators that alter immune system sensitivity. It is clear that GH exerts stimulatory effects in central and peripheral lymphoid organs being considered a potent immunomodulatory hormone (Auernhammer, 1995). All hematopoietic lineages found in bone marrow, including B lymphocytes, macrophage, T lymphocytes and granulocytes precursors, express GH receptors to various extents (Dardenne et al., 1998). Several studies have demonstrated that, exogenous GH may improve a variety of immune functions including B lymphocytes responses and antibody production (Kimata, 1994), NK activity (Stephenson et al., 1991), macrophage activity (Gaytan et al., 1994), T lymphocytes function and neutrophil function (Fu et al., 1992). See Figure 1.

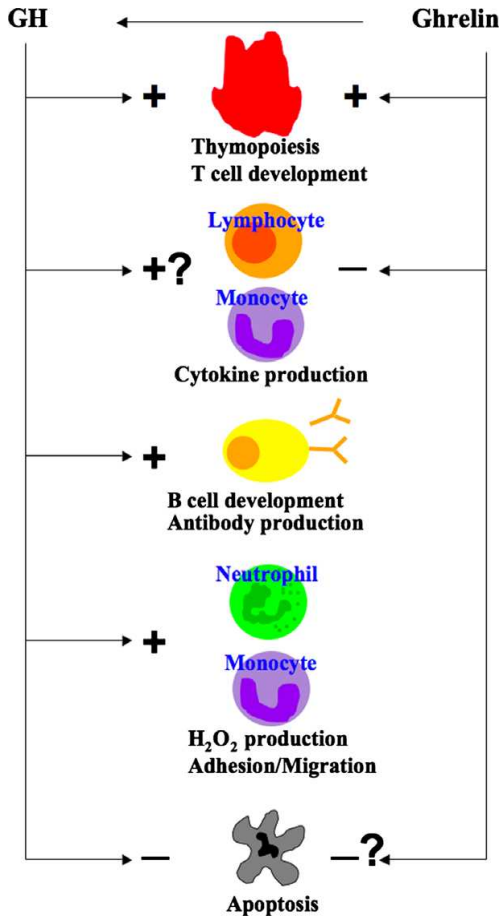


Figure 1. Growth hormone and ghrelin in the immune system (Hattori, 2009).

2.9 GH receptors and expression on immune cells

Growth hormone binding protein (GHBP) first reported in 1964, corresponds to the extra cellular domain of the GHR (Kelley and colleagues., 2007). In IM-9 lymphocytes, GHBP are formed through proteolysis and it is suggested that it shows antagonistic properties *in vitro* and agonistic in vivo (Jones & Clemmons, 1995). It was reported that human lymphocytes secrete GH releasing hormone (GHRH) and IGF-I expressing their respective receptors (Weigent, 1991). The affinity constant of GHR, by the Scatchard analysis is 1.3-1.5 x 10^{-9}l/mol and the receptor numbers is usually from 4000-7000 per cell (Cunningham et al., 1990; Fu et al., 1992). The presence of GHR on peripheral blood mononuclear cells can be obscured by the fact that GH can bind to prolactin receptors as well (Cunningham et al., 1990; Fu et al., 1992).

As previously evaluated, GH may have a multifactor influence in the immune cells number and function and this project aims to evaluate these effects in healthy human population.

2.10 Haemorheology

Haemorheology is the science that describes the flow characteristics of the blood (El Sayed et al., 2005). Essential delivery substrates in cells and the removal of the by products of cell metabolism are related to microcirculation and are influenced by the microvascular network (Kamm, 2002). Erythrocytes form the majority of the blood constituents (up to 98%); hence any change in their flow behaviour affects the blood flow through the cardiovascular system and consequently the blood circulation (Sanjay, 2002).

The properties of erythrocytes include erythrocyte deformation by shear force reducing the flow resistance and erythrocyte aggregation resulting from an increase in flow resistance (Kamm, 2002).

A relationship between GH and the rheologic function of circulating RBC was suggested by Engstrom and colleagues (1990). After analysing hypophysectomy rats with GH treatment (1mg/kg/day continuously infused in female rats during 21 days) RBC deformability and filterability were normalised (Engstrom et al., 1990), suggesting a relationship properties between GH and blood rheology. Importantly, GH has been shown to induce erythropoiesis (Brun, 2002). It is these limited studies that indicate GH may play an important role in influencing haemorheology, however RBC aggregation and deformability have not been determined.

2.10.1 Erythrocyte Aggregation

Erythrocyte aggregation is one of the most important factors which determine the blood flow at low flow shear rates in the microcirculation (Skalak, 1981). The microcirculation helps to

regulate the O_2 supply to the tissues which can be consequently influenced by the aggregation of the erythrocytes. The growth process of the structure of the aggregates depends on the deformation of the shape of the outermost cell (concave or convex) available for attachment to individual erythrocytes (Skalak, 1981). Red blood cells (RBC) in the presence of plasma proteins, principally fibrinogen, may aggregate to form rouleaux formations (Shalak, 1981). Of particular importance are the rheological properties of erythrocytes, since erythrocytes constitute for up to half of the volume of the blood (Maeda, 1996).

The extent of RBC aggregation is determined by opposing forces: the repulsive force between the negatively charged cells, the cell-to-cell adhesion induced by plasma proteins, and the disaggregating shear force generated by blood flow (Chien, 1982). Red blood cell aggregation is thus dependent on both plasma factors and on cellular factors. It is agreed that the blood flow is sufficient for dispersion of RBC aggregates (Chien 1982). It is assumed that this process is dependent on both the size of RBC aggregates and the cohesive forces within aggregates, expressed by the shear stress required to disperse them.

Red blood cell aggregation is increased in various conditions associated with an inflammatory response (Weng, 1996). At low shear rate, an increase in red cell concentration promotes red cell aggregation, which increases effective cell volume and blood viscosity (Skalak, 1984). At high shear rate, an increase in red blood cell concentration promotes deformation of red cells, which decreases effective cell volume and hence compensates for the increase in viscosity (Lowe, 1980). Claustres and co-workers (1987) demonstrated the delivery of substrate and O_2 to exercising muscle could be increased by the administration of GH and IGF-I, suggesting a possible interaction of GH and haemorheology, however further studies need to be conducted to assess the effect of administration of GH on blood rheology of healthy patients. This link between body composition, haemorheology and GH can be

justified due to the GH being considered a major regulator of body content in fat and water (Brun, 2002).

Aggregation of RBCs can be quantitatively measured by evaluating the RBC sedimentation rate (Houbouyan et al., 1998), zeta sedimentation rate (Bull & Brailsford, 1972), low-shear viscometry (Baskurt & Meiselman 1997), using a flow chamber analysis (Chen et al., 1994), ultrasound back scattering (Boynard et al., 1987) and photometry analysis (Hardeman et al., 1994). The Myrenne aggregometer is a type of back scattering instrument used in determining RBC aggregation. This instrument simultaneously disseminates pre-existing aggregates via cone rotation in a transparent cone-plate chamber at a shear speed of 600seg-1/10sec (Vaya et al., 2003). Two mode measurements are generated from this instrument relating to the speed of rotation of the cone at stasis (M) and at $3s^{-1}$(M1) (Vaya et al., 2003). The increase in aggregation corresponds to an increase in these two indices (Vaya et al., 2003). The results can either be recorded after 5 or 10 seconds after the cone has halted its rotation (Vaya et al., 2003).

2.10.2 RBC Deformability

Cell deformability is characterised by general measurement of cell biology and physical forces applied to the red blood cell that influences macro- and microcirculation (Musielak, 2009). Growth hormone, IGF-I and IGF-II are shown by early studies to stimulate erythropoiesis (Golde, 1977; Fruhman, 1954). Additionally GH-IGFI axis has great effects on body composition, fuel and fluid metabolism and it is likely to influence the viscosity of blood, consequently, RBC rheology, (Brun et al., 2002) and consequently performance of athletes.

The close observation of RBC deformation requires the development of certain instrument that measure deformability, for this reason instruments such as the micropore filtration, high-

speed centrifugal deformation, micropipette aspiration and the Rheoscope have been developed (Pfafferott et al., 1985). The most extensively used techniques however are RBC filtration, ektacytometry and Rheoscope (Shin et al., 2004) which quantifies pressure across the RBC membrane or the time it takes for a particular amount of RBC to move through a vessel (Shin et al., 2004). One limitation with this method is the lack of a standard with which to compare the measured values (Shin et al., 2004).

The rheoscope conversely deforms RBC through the application of shear flow forces (Dobbe et al., 2002). It employs the use of the microscope and counter rotating plates on which the RBCs are placed (Dobbe et al., 2002). Images are projected on to a video camera and adjusted using both bright field stroboscopic illumination and an interference filter (Dobbe et al., 2002). The apparatus, however, is still quite cumbersome and requires great operator skill (Mohandas et al., 1980).

The Ektacytometry on the other hand applies laser diffraction procedure to RBC while subjecting them to various types of stresses from a small amount of blood (Bessis & Mohands 1980). Rheoscan-D is presently the latest version of ektacyometer being used to measure deformability in erythrocytes. The Rheoscan-D is a slit ektacytometer having a laser, a video camera, a pressurised slit medium and a vacuum generating apparatus (Shin et al., 2005). A suspension of blood is placed in a sample chamber which allocates the blood in to the slit (Shin et al., 2005). In the slit a laser beam is transmitted which becomes diffracted once it comes into contact with the RBCs (Shin et al., 2005). As pressure in the slit compartment decreases or increases the RBC change their conformation (Shin et al., 2005).

2.10.3 Fibrinogen

Fibrinogen is a 340 kDa dimeric glycoprotein composed of three pairs of non-identical polypeptide chains denoted Aα, Bβ, and γ (Henschen and Lottspeich, 1977). Previouly

described by Reinhart (2003) fibrinogen is required in the final step of the coagulation cascade and transformed into fibrin through thrombin. Furthermore, fibrinogen is considered a major determinant of plasma viscosity and erythrocyte aggregation (Reinhart et al., 2003). Furthermore, fibrinogen has been considered the major plasma protein coagulation factor (Lowe et al., 2004). In Larsson and colleagues (1984) during 13.5 years of follow up with almost 800 men aged 54, fibrinogen level was suggested to have an important role in the development of stroke and myocardial infarction for its coagulations effects.

2.11 Animal studies with GH

Studying animals can represent a model for the investigation of the relation of GH and immune system. Growth hormone plays an important role in the regulation of humoral and cellular immune responses due to existence of GH receptors (GHR) on immune cells such as B, T lymphocytes and macrophages (Murphy et al., 1992). This has been observed in GH deficient animals by several studies (Gala et al., 1993). Growth hormone receptors are expressed on immature $CD4^{-}CD8^{-}$ double negative and $CD4^{+}CD8^{+}$ double positive thymocytes, suggesting that GH could have a direct influence in the early stages of T lymphocytes differentiation, thus confirming the role of GH hormone in T lymphocytes development (Gagneraud et al., 1993). Growth hormone receptors belongs to the cytokine-GH-PRL receptor superfamily, which includes receptors for many cytokines involved in the growth and differentiation of lympho-hematopoietic lineages, and GHR could mediate similar effects (Gagneuraud et al., 1993).

Seiva and colleagues (2008) using 25 male Wistar rats with heart failure, applied two different doses of rhGH (1mg/kg/day and 2 mg/kg/day) subcutaneously for two weeks and concluded that, GH effects dose-dependent with both tested doses which did not affect the

heart dysfunction. The higher GH dose, 2 mg/kg, exerted detrimental effects related to energy metabolism and oxidative stress, while the smaller dose, 1 mg/kg GH, exerted beneficial effects; enhancing antioxidant defences, reducing oxidative stress and improving energy generation in myocardium of rats with heart failure.

As shown in previous animal studies mentioned (Gala et al., 1993; Gagneraud et al., 1993) GH may affect the function of immune cells and erythropoiesis. This is the first study to evaluate those effects in healthy human subject's immune cells and erythrocytes.

2.12 Gene expression

The primary step in gene expression is the transformation of deoxyribonucleic acid (DNA) to messenger ribonucleics acid (mRNA) through the process of transcription. This is then followed by the conversion of the mRNA information to proteins in a cellular process known as translation (Orphanides & Reinberg 2002). Messenger RNA (mRNA) is the link between DNA and proteins whereby measuring the change in transcription level between treated vs non-treated states has become an increasingly useful tool used to study gene transcription and expressional changes leading to a better understanding of biological processes (Bustin et al., 2004).

Regulation of gene expression is a necessary component of optimal eucaryotic function. This can be achieved by adjusting protein activity, generating protein diversity and controlling the concentration of a protein in a particular area (Orphanides & Reinberg 2002). These procedures are necessary for intracellular signalling via phosphorylation of proteins, metabolic functions of various proteins and cellular activation (Orphanides & Reinberg 2002).

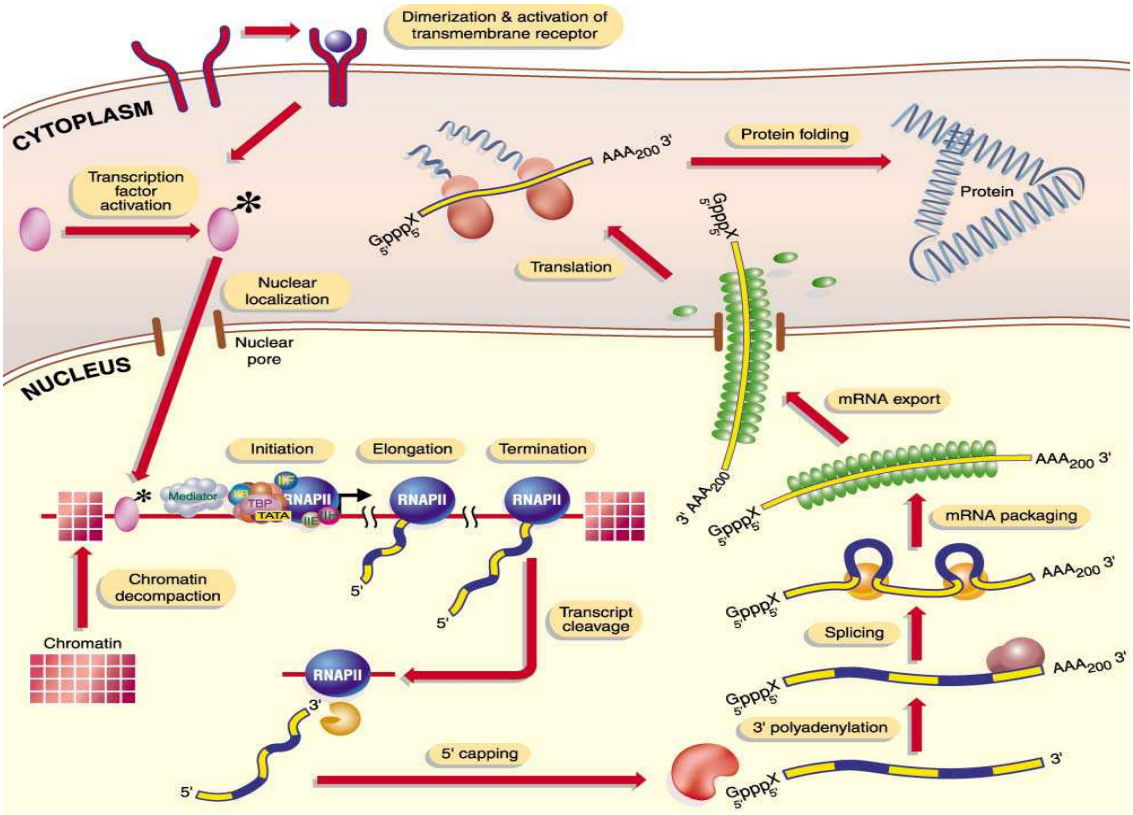


Figure 2: The process of transcription and translation (Orphanides & Reinberg 2002).

White blood cells (WBCs) express tens of thousands of genes, whose expression levels are modified by genetic and external factors. A number of authors (Maas et al., 2002; Whitney et al., 2003; Baechler et al., 2004; Gregersen 2003) have recently explored the use of peripheral blood cells as a readily available source of material for gene expression analyses in human. The mechanism by which inducers of drug metabolism and drug transport exert their effects in humans in vivo is poorly defined because sampling of tissues of interest, such as the liver, is by necessity restricted. However, use of the human lymphocyte, a readily obtainable blood fraction may be valuable in quantifying induction events on an individual basis. This study will consider PBMC's for evaluating the effects of rhGH on the expression of genes associated with the immune system in healthy young males. This investigation will use real-time RT-PCR to monitor the expression pattern of selected genes in human haematopoietic cells exposed to rhGH. The potential application of cDNA microarray and/or quantitative

RT-PCR (Q-RTPCR) analyses, using samples obtained from leucocytes has been recognised, and provides theoretical support for this approach in the context of selected immune genes expression after short-term administration of rhGH in healthy subjects. On the RT-PCR the reverse transcription is the first step, the exponential amplification via RT-PCR provides a highly sensitive technique where a very low copy number of RNA molecules can be detected (Chelly et al., 1989)

2.13 Genes related to GH

In the present study the aim was to determine if selected genes related to the activity of immune system (IL6, IL6R, IL10R, PFR1, NFKB and GZMA) and their regulation are influenced by short-term GH administration. As there are speculations of the use of rhGH by healthy young males, it is important to further investigate the cellular effects of rhGH on the immune function at the cellular level of healthy young males.

The protein encoded by Perforin (PFR1) has structural and functional similarities to complement component 9 (C9). Like C9, this protein creates transmembrane tubules and is capable of lysing non-specifically a variety of target cells. This protein is one of the main cytolytic proteins of cytolytic granules, and it is known to be a key effector molecule for T-cell- and natural killer-cell-mediated cytolysis. According to Liu et al., (1989), PFR mRNA was detected in most T blast populations.

Nuclear factor kappa B (NFκB) is a protein complex that controls the transcription of DNA and is found in almost all animal cell types. It plays a key role in regulating the immune response to infection. Through a cascade of events, the kinase complex is activated and NFκB is able to enter the nucleus to up regulate genes involved in T cell development, maturation, and proliferation. Thus, evaluation of the expression of NFκB up or down-regulation may generate indication of the influence of the rhGH in the athlete immune function.

Interleukin 6 (IL6) encodes a cytokine that functions in inflammation and the maturation of B cells (Yasukawa et al., 1987). The protein is primarily produced at sites of acute and chronic inflammation, where it is secreted into the serum and induces a transcriptional inflammatory response through interleukin 6 receptor. Its receptor system consists of two molecules: a ligand-binding molecule and a non-ligand-binding signal transducer, both part of the cytokine receptor family (Omoigui, 2007).

Interleukin 10 Receptor (IL10R) is a heterotetramer composed of 2 of each of the receptor chains which belong to class II cytokine receptors (Liu et al., 1994). Interaction of IL10 with the IL10R complex stabilizes dimerization of IL10R subunits, activates phosphorylation of the receptor-associated Janus tyrosine kinases, Janus kinase (JAK) 1 induces signal transducers and activators of transcription (STAT) 3- and STAT1-mediated signal transduction as described by previous studies Riley et al., (1999) and Donnelly et al., (1999).

Granzyme A (GZMA) is located in the granules of cytotoxic T and NK cells. The pathway of T lymphocyte cytotoxicity was described by the perforin/granzyme pathway (Valerio et al., 1997). In data, in vivo analysis of cytotoxic CD4+ T cells indicates that they have lytic granules containing cytotoxic factors such as granzymes and perforin (Appay, 2004). It was shown in the literature that CD8+ cytotoxic lymphocytes and NK cells use the perforin/granzyme pathway as a major mechanism to kill pathogen-containing cells and tumor cells (Shresta et al., 1998).

2.14 Significance

This is the first study to evaluate the possible effects of short-term administration of rhGH to healthy young males on the rheology of the blood as it is a great influence in the status of the delivery of substrates as well as O_2 to the tissue and consequently a compulsory factor for the characterization of the healthy status of the blood flow.

According to the "open window" theory (Nieman, 2000), the immune system of athletes may vary due to training and competitions status. Since there is a lack of information on how exogenous rhGH can possibly affect the immune numbers and function of healthy population this study become mandatory for such understanding.

This research had particular significance in providing evidence for changes in immune cells and function, due to the short-term administration of rhGH in healthy subjects. Growth hormone binding was first detected on a human B cell lymphoma (IM-9) lymphocyte cell line (Lesniak et al., 1974) and subsequently on human PBMCs (Lymphocytes and monocytes) (Kiess et al., 1985) as such this study investigated whether changes in gene transcription within PBMCs provide evidence on the possible effects of the use of rhGH in healthy young males. This is a stringent study that considers rhGH effect on lymphocytes number and function, haemorheology and gene expression in healthy active adults.

The objectives of this project were to:

1. Assess possible alterations in blood rheology due to short-term recombinant human GH (rhGH) administration to evaluate the effects in aggregation, deformability, plasma fibrinogen and IGF-I levels in healthy young males

2. Evaluate the immunomodulatory effects of short-term administration of recombinant human GH (rhGH) in healthy young males in relation to NK cell number, activity and

phenotype, T cell number, cytokine production of IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ and $CD4^+/CD8^+$ ratio

3. Evaluate the effects of short-term administration of recombinant human GH (rhGH) in healthy young males on the gene expression of selected genes whose activity is associated with the function of immune system (IL6, IL6R, IL10R, PFR1, NFKB and GZMA).

Chapter 3

Research studies of this thesis

3.0 Research studies of this thesis

The following chapter describe the studies conducted aiming to answer the stated research questions.

3.1 Study One

The following paper was published in the Journal of Clinical Haemorheology and Microcirculation 2010 (ISSN 1386-0291). Study one evaluated the hemorheological effects of short-term administration of human recombinant growth hormone (rhGH) in healthy young males. The study one also correlated the hemorheological parameters evaluated to IGF-I in serum.

3.2 Study Two

Study two was published in the European Journal of Applied Physiology, Nov 22, 2010. The purpose of the following study was to evaluate the immunomodulatory effects of short-term administration of recombinant human GH (rhGH) in healthy young males. NK cell number, activity and phenotype, T cell number, cytokine production of IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ and $CD4^+/CD8^+$ ratio. The study also evaluated the results with IGF-I production by RIA.

3.3 Study Three

The study three was accepted for publication in the Doping Journal and the aim of this study was to evaluate the effects on the gene expression of selected immune genes (IL6, IL6R, IL10R, PFR1, NFKB and GZMA) after short-term administration of rhGH.

Study One

The effects of short-term recombinant human growth hormone (rhGH) administration on blood rheology in healthy young males

S.B. Ramos[1], E.W. Brenu[1], J. F. Brun[2], B. Gray[1], S. Rogerson[1], R.P. Weatherby[3], L. Tajouri[1], S.M. Marshall-Gradisnik[1]

1- Faculty of Health Sciences and Medicine
Bond University, Robina
Queensland, Australia

2- Batiment Crastes de Paulet,
Hôpital Arnaud de Villeneuve,
Montpellier cedex 5, France

3- Department of Exercise Science and Management
Southern Cross University,
New South Wales, Australia

Contact information: Sandra Ramos
Faculty of Health Sciences and Medicine
Bond University - Gold Coast
Queensland 4229, Australia
Phone 07 5595 1654
e-mail: sandra.ramos2@student.bond.edu.au

Abstract

Introduction: It has been shown that growth hormone (GH) exerts regulatory effects on haemorheology and other metabolic functions. Growth hormone stimulates the production of IGF-1 and GH–IGF-I system has profound effects on body fluid status. There is speculation that GH has become widely used as a performance enhancing drug among athletes of various sports. The present study evaluated the possible hemorheological effects of short-term administration of human recombinant growth hormone (rhGH) in healthy young males. **Methodology:** 30 young healthy males (27 ± 9 years) participated in a 29 day study where they were administered either 0.9% sodium chloride or 1mg of human rhGH from day 1 to day 7. The participants were randomly assigned into either placebo (C) n=15 or rhGH 1mg/day (rhGH) group n=15. This study evaluated plasma fibrinogen levels, red blood cell (RBC) aggregation and deformability and serum insulin-like growth factor I (IGF-I) level between and within the groups along 29 days. **Results:** There was a significant increase in erythrocytes aggregation index post rhGH administration (day 8), in accordance to an increase in IGF-I level in serum.

Key words: rhGH; aggregation, deformability, fibrinogen, IGF-I.

1. Introduction

Human growth hormone (hGH) is considered a pleiotropic hormone that is essential for the stimulation of many metabolic processes in cells (Barroso et al., 2008). For the first time in the sport's history during the 2004 Olympic Games in Athens athletes were tested for the presence of recombinant human growth hormone (rhGH) in competition. In total, 691 blood specimens were tested for rhGH as well as haemoglobin-based oxygen carriers (HBOCs) and homologous blood transfusion (BT). All samples analysed for rhGH (397 in total) were reported negative as there is not a test for rhGH able to detect it after a short period (24 h) of time after administration of the hormone. Recombinant human growth hormone is described as the most expensive, most fashionable and least understood of the new athletic drugs (Holt and Sonksen, 2008), rhGH alone or in combination with other performance enhancers has been considered the prevailing drugs of choice (Holt and Sonksen, 2008).

The rheological properties of plasma and blood cells are markedly influenced by hormones (Brun, 2002). Growth hormone and insulin-like growth factor I (IGF-I) have been demonstrated to stimulate erythropoiesis *in vitro* (Golde et al., 1977) and in animal models (Peschle et al., 1972).

The affects on morphology and filterability of circulating red blood cells (RBCs) between GH and the rheologic function of circulating RBC has been suggested by Engstrom and colleagues (1990) using hypophysectomised rats (Engstrom et al., 1990). Growth hormone treatment (1mg/kg/day continuously infused in female rats during 21 days) was shown to normalise RBC deformability and filterability. Importantly comparable, GH has been shown to induce erythropoiesis (Brun 2002) as erythrocytes express specific receptors for IGF-I and II (Polychronakos et al., 1983). However, whether administration of rhGH has a regulatory effect on blood rheology in healthy young males is not known.

Furthermore GH is able to increase Total Blood Volume and stimulate erythropoiesis in adults with growth hormone deficiency (Christ et al., 1997). Administration of GH (0.036 U/kg·day) increased mean IGF-I values to within the normal range. GH treatment significantly increased RBC number at 3 months with no change in the placebo group (Christ et al., 1997).

Plasma proteins such as fibrinogen concentration promote RBC aggregation being considered the most important determinant of the aggregating property of plasma (Rampling 1988) and it affects blood viscosity both at high shear rate (primarily relevant for flow in arteries, arterioles, and capillaries) and low shear rate (relevant for flow in veins and under stasis) (Vayá et al., 2009).

It can be implied that the delivery of substrate and O_2 to muscle can be increased by the administration of GH and IGF-I (Luquita et al., 2009), highlighting an interaction between GH and haemorheology. However, further studies need to be conducted to assess the effect of administration of GH on blood rheology of healthy adults. The aims of the study were to evaluate the effects in aggregation, deformability, plasma fibrinogen and IGF-I levels in healthy young males due to short duration administration of rhGH.

2. Material and Methods

Healthy males (n=30) were randomly assigned into two groups: treatment and placebo. Daily injections of either rhGH (1mg) or placebo (0.9% sodium chloride) were intramuscularly administrated for 7 days. Prior to each injection all subjects received a standard breakfast (protein shake Body Science, Australia). Subsequent to the injections, collected blood samples from all participants were assessed for aggregation, deformability and plasma fibrinogen prior to (day 0), post seven days injection (day 8).

Blood samples (50mL) were collected, via venipuncture of an antecubital vein into ethylenediaminetetraacetic acid (EDTA), serum separator tubes (SST) and heparin tubes (Becton Dickinson, USA).

2.1 Subjects

Thirty healthy young males were recruited and randomly divided into two groups. rhGH (n=15, 24.5 ± 6.5 y.o.) for the treatment group and Placebo (n=15; 26.1 ± 6.6 y.o.). Volunteers were excluded on the basis of the following conditions: (a) regular use of therapeutic and/or recreational drugs, (b) contraindications or risk to exercise (recent injuries/risk factors for maximal strength testing). Selected subjects were required to read and sign an informed consent form prior to the experiment. This study was approved by the Bond University Human Research Ethics Committee.

2.2 Erythrocyte Aggregation

The Myrenne aggregometer (light-transmission technique) was used to determine erythrocyte aggregation by methods previously described (Marossy et al., 2009), where erythrocytes aggregation was assessed at 'M' (aggregation during stasis after shearing at 600 s^{-1}) and 'M1' (facilitated aggregation at low shear rate after shearing at 600 s^{-1}). Erythrocyte aggregation tests were performed in native blood immediately after collection, haematocrit (HCT) adjusted autologous plasma solution as well as in standardised HCT adjusted 3% Dextran-70 solution at a 40% HCT to account for variation between subjects.

2.3 Erythrocyte Deformability

Erythrocyte deformability was determined by methods described by Shin and colleagues (2005). Briefly it consists of laser diffractometry in the Ektacytometer (Technicon, France) which measures the ellipticity of the diffraction image produced by a laser beam as the red

cells elongate under rotational shear. An elongation index is calculated from the light intensity along the major and minor axes of the diffraction image.

2.4 Plasma Fibrinogen

Plasma fibrinogen concentration was determined quantitatively using the Von Clauss clotting method (Clauss, 1957). It measures the rate of fibrinogen to fibrin conversion in diluted sample under the influence of excess thrombin (Clauss, 1957).

2.5 IGF-I serum concentration

The double antibody radioimmunoassay system (RIA) was applied for quantitative *in vitro* diagnostic measurement of IGF-I in serum by a commercial RIA (IGF-I kit – Bioclone – Australia). IGF-I is firstly separated from its binding protein in plasma then, the analyte competes with ^{125}I labelled tracer antibody for binding to a constant amount of antibody. A second antibody coupled to magnetisable polystyrene particles is used to separate antibody bound from free ^{125}I labelled tracer antibody.

2.6 Statistics

The statistical analyses were performed using SPSS software, version 17.0. ANOVA was applied and the differences were considered statistically significant when $p \leq 0.05$. Bonferroni was the post hoc test applied.

3. Results

Table 1. Subjects characteristics (mean±SD).

Group	N	Height (cm)	Weight (kg)	Age (years)
Placebo	15	178.9±5.9	83.1±9.1	26.1±6.6
rhGH	15	178.8±9.5	81.2±17.9	24.5±6.5

Haematocrit

Haematocrit (HCT) was determined using a full blood count from the Coulter Counter (Table 2). Samples had the HCT adjusted to 40% either by removing or adding autologous plasma.

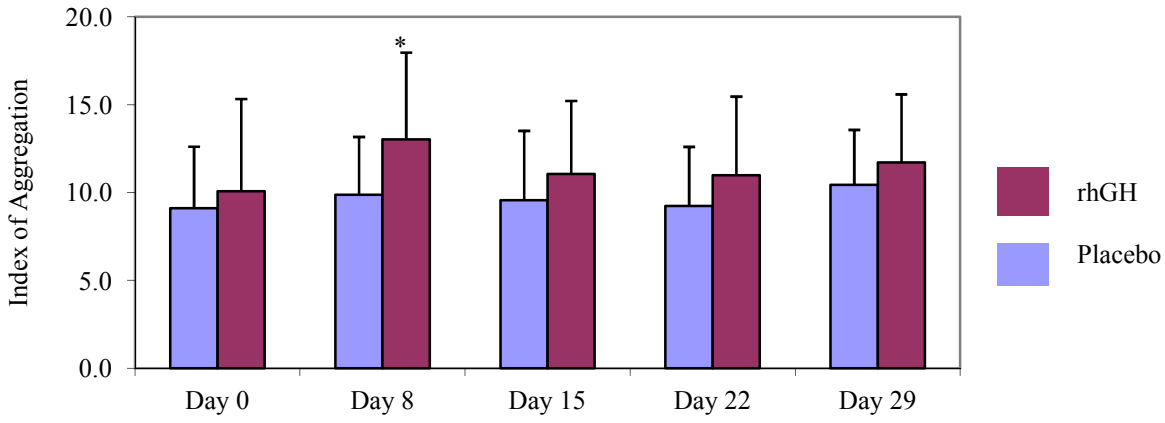
Table 2. Haematocrit (%) of groups before being adjusted for aggregation measurement.

Group		HCT_day 0	HCT_day 8	HCT_day 15	HCT_day 22	HCT_day 29
Placebo	Mean	42.30	41.52	42.98	42.65	41.57
	SEM	0.89	0.66	0.58	0.74	0.87
rhGH	Mean	42.76	42.48	42.91	42.73	42.59
	SEM	0.93	0.65	0.55	0.61	0.54

Note: HCT adjusted 3% Dextran-70 solution at 40% HCT

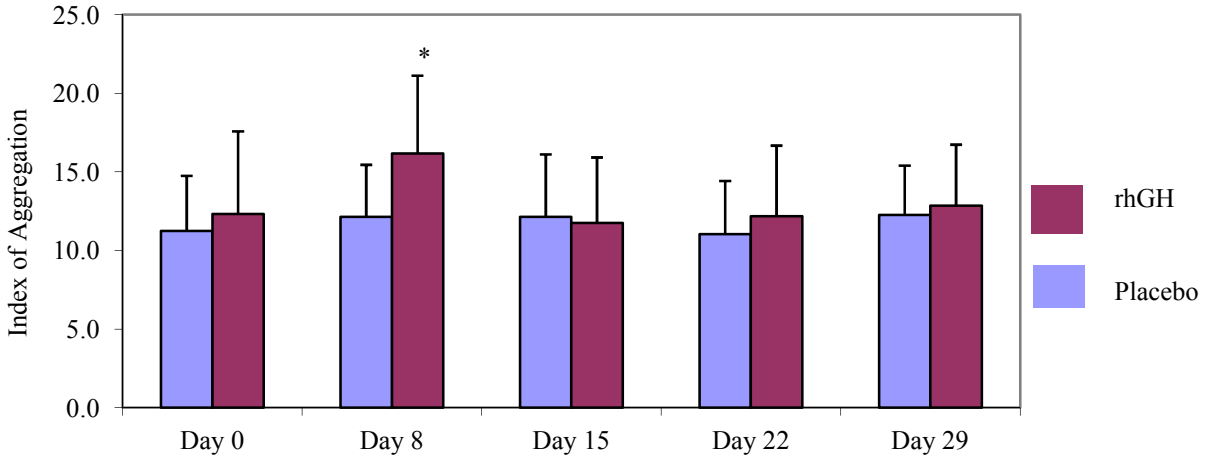
Erythrocyte aggregation

Erythrocyte aggregation at indexes "M" and "M1" demonstrated significant difference from baseline (week 1) to day 8 (week 2) between groups and within group (rhGH).



* Significant difference between day 8 and days 0, 15, 22 and 29 for $p \leq 0.05$.

Figure 1. Erythrocyte aggregation index of whole blood at "M" from baseline (day 0) to day 29.



* Significant difference between day 8 and days 0, 15, 22 and 29 for p≤0.05.

Figure 2. Erythrocyte aggregation index of whole blood at "M1" from baseline (day 0) to day 29.

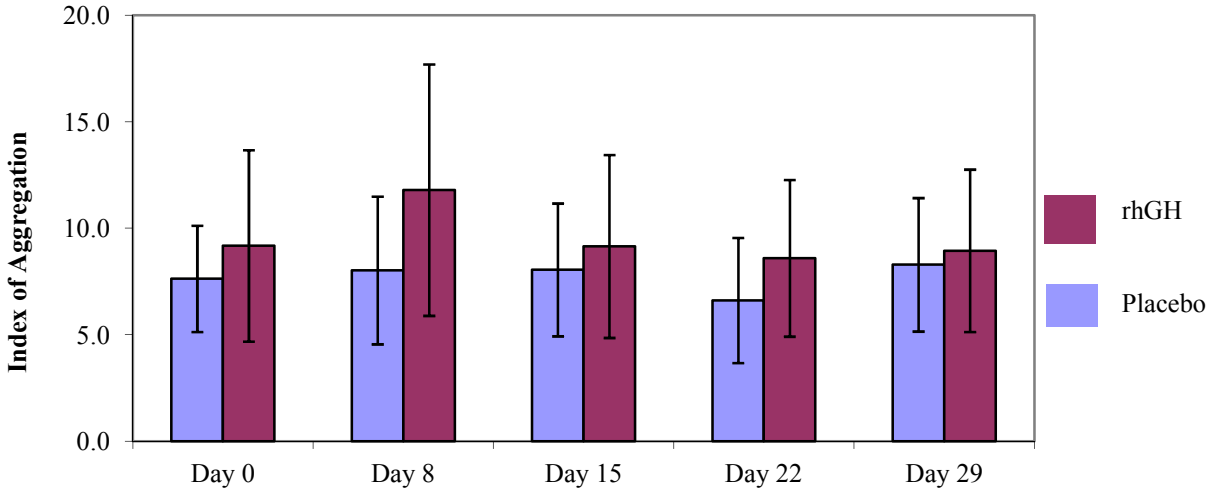


Figure 3. Erythrocyte aggregation index at "M" after been adjusted for HCT 40%.

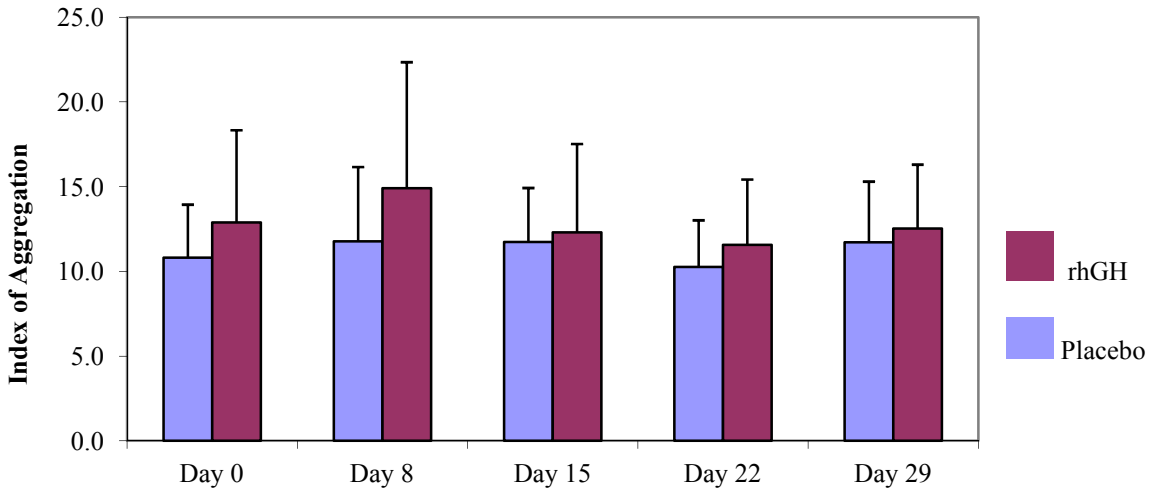
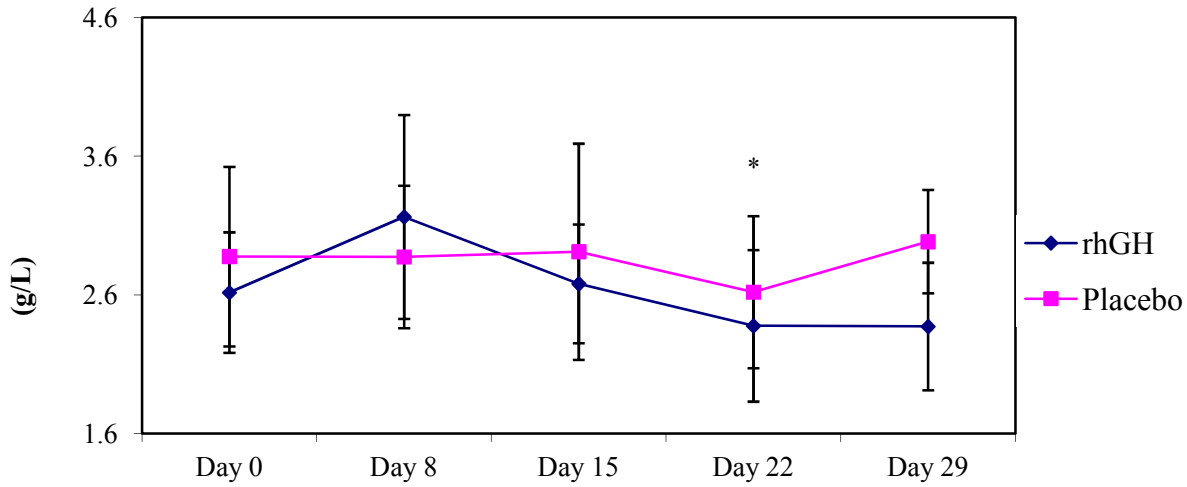


Figure 4. Erythrocyte Aggregation index at "M1" after adjustment for 40% HCT.

Fibrinogen

Initial measurements of fibrinogen were determined for baseline at day 0 and both groups showed similar results. Fibrinogen presented no statistical difference between groups at day 8 after subjects had received 7 days of either 1 mg/day of rhGH or 0.9% sodium chloride injections. After a slight increase in plasma fibrinogen mean in the rhGH group (Fig.1) from baseline to day 8 (3.10 to 3.61 g/L), results were levelled off at day 15 showing no difference between groups compared to baseline (Day 0).



* Significant decrease ($p \leq 0.05$) in plasma fibrinogen from day 8 to day 22 in the rhGH group.

Figure 5. Plasma fibrinogen (g/L) measurements from baseline (day 0) to day 29.

Deformability

In the rhGH group the deformability was not significantly different from the Placebo group along the days of the study (Fig. 6).

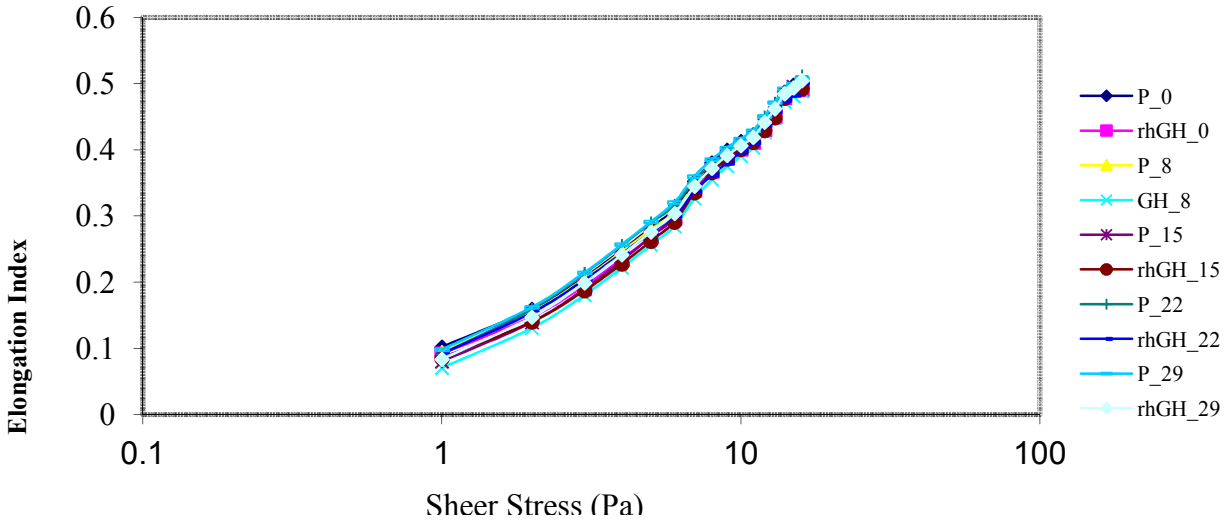
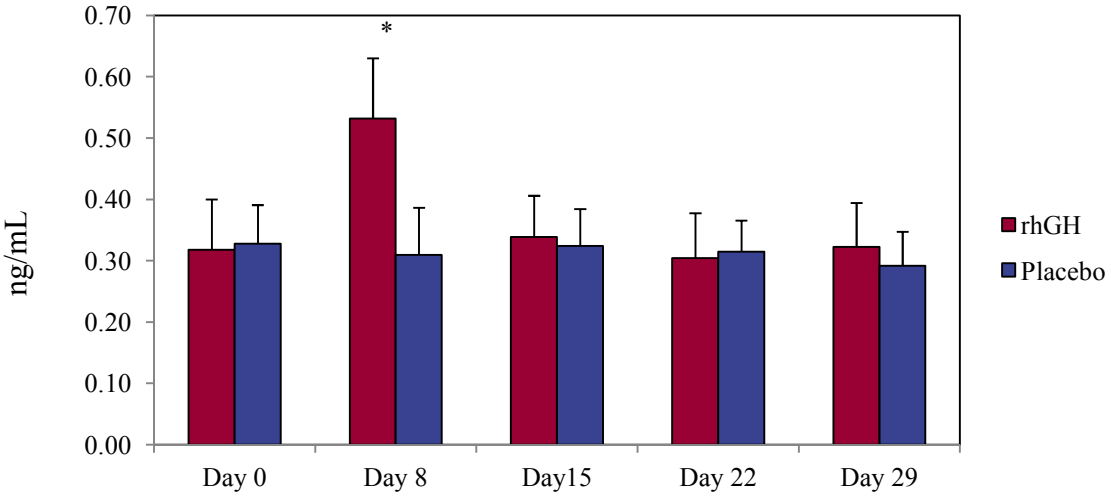
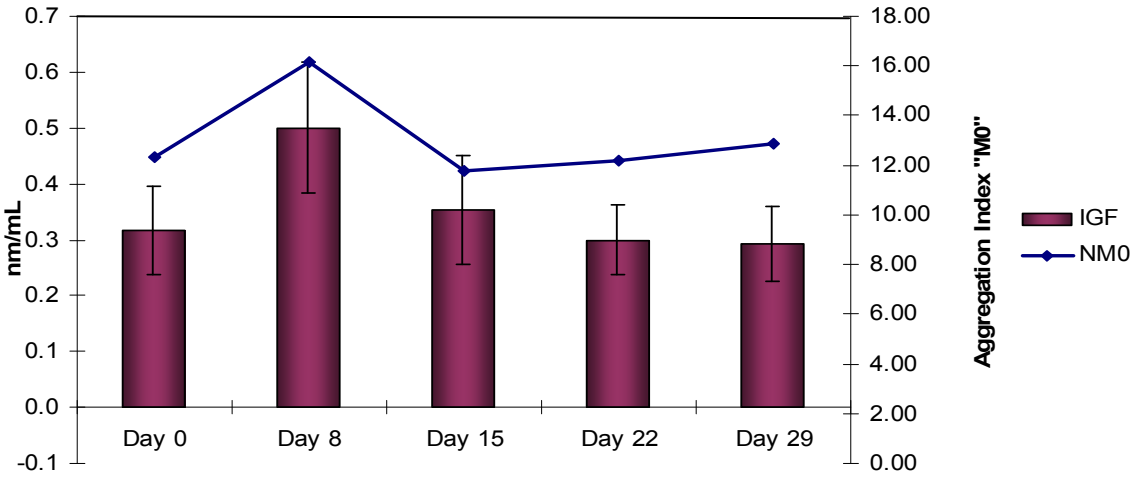


Figure 6. Deformability from day 0 to day 29 for Placebo and rhGH groups.



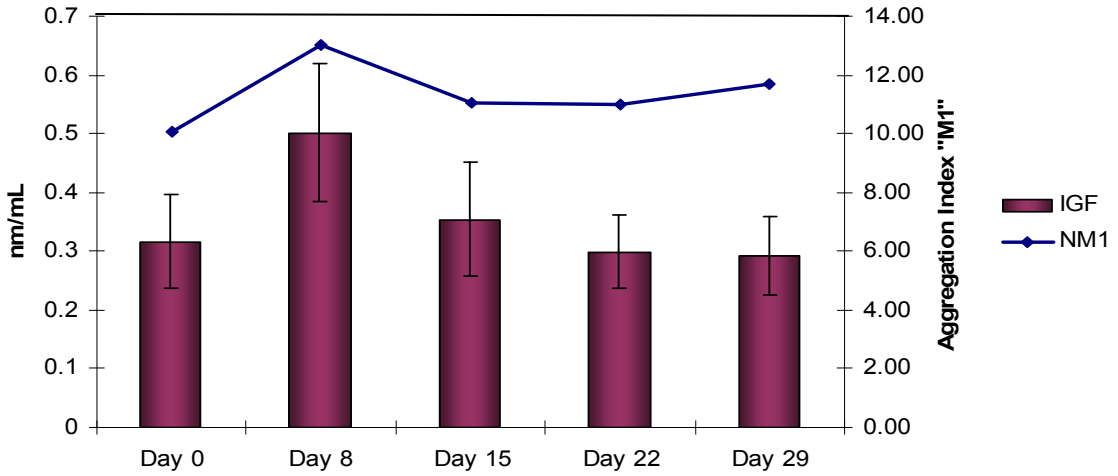
* Significant difference between day 8 and days 0, 15, 22 and 29 for p≤0.05.

Figure 7. IGF-1 analysis in plasma per collection day (0 to 29) for groups.



* Significant correlation Pearson p≤0.05.

Figure 8. Correlation of erythrocytes Native "M" (NM) aggregation index with IGF-I in serum from day 0 to day 29 of the study.



* Significant correlation Pearson p≤0.01.

Figure 9. Correlation of erythrocytes Native "M1"aggregation index with IGF-I in serum from day 0 to day 29 of the study.

4. Discussion

The study aimed to evaluate possible alterations in blood rheology and IGF-I levels in healthy young men after short-term (7 days) administration of 1mg of rhGH compared to (C) healthy subjects. The results of this study demonstrated that 7 days of rhGH (1mg) administration induced significant alterations in the blood rheology as well as in IGF-I concentration in the serum of healthy young males.

Fibrinogen is the major determinant of plasma viscosity and erythrocyte aggregation (Clauss, 1957). It affects blood viscosity at high shear rate (primarily flow in arteries, arterioles, and capillaries) and low shear rate (in veins and under stasis) (Clauss, 1957). Although plasma fibrinogen had an increase of 0.55 g/L from baseline to second week in the rhGH group, it was not statistically different from baseline. Perhaps this showed to be enough to induce a significant increase in the index of aggregation in the rhGH group from baseline to day 8.

Native aggregation (whole blood) of erythrocytes was significantly increased on day 8 in the rhGH group, after receiving 7 days of rhGH injections. However, the results for aggregation index at day 29 showed a significant decrease compared to day 8 indicating an acute effect of the hormone.

While there are GH receptors in the bone marrow, *in vitro* experiments using human erythroid progenitors have shown that the role of GH in erythropoiesis is probably mediated by IGF-I resulting in potential proliferation in erythroid progenitors (Philipps et al., 1988). IGF-I in serum increased significantly 24 h after rhGH administration $p \leq 0.01$ and remained constant for the placebo group.

The GH–IGF-I axis is a regulator of blood rheology and in physiological conditions such as regular training in athletes it has the potential to alter blood rheology factors (Peyreigne et al., 1998). GH and IGF-I, together with other hormones and growth peptides, modulate the formation and functional activation of mature blood cells. GH appears to have multiple direct and indirect effects on the human fetal bone marrow compartment by facilitating the proliferation and maturation of multilineage progenitor cells (Hanley et al., 2005).

Significant correlations were established between increase in Native aggregation indexes and IGF-I serum for the rhGH group (0.45 and 0.48 for NM $p \leq 0.05$ and NM1 $p \leq 0.001$ respectively). Levelling off on day 15 of the study, IGF-I levels due to rhGH injections presented acute effects on blood rheology, mainly on native aggregation. Previously, IGF-I had shown to be correlated with blood viscosity in 39 healthy males (elite sportsmen, age 23.7±0.72 years) on physical training, when in the upper quintile IGF-I levels were associated with an increase in erythrocyte aggregation, and increased viscosity of the blood (Monnier et al., 2000).

Whether the reported increase in erythrocytes aggregation indices and IGF-I in serum can lead to any improvement or decrement in sports performance is still to be studied.

6. Conclusion

The results from this study indicate 1mg/day of rhGH for seven days may adversely affect blood rheology. Whether users may be more liable to higher viscosity of blood highlighting potential adverse health effects of the use of rhGH needs investigation.

7. References

See page 132.

Study Two

Assessment of immune function after short-term administration of recombinant human growth hormone in healthy young males.

S.B. Ramos[1,2], E.W. Brenu[1,2], R. Christy[1], B. Gray[1], L. McNaughton[1], L. Tajouri[1], M. Van Driel[1], S.M. Marshall-Gradisnik[1,2]

1- Faculty of Health Sciences and Medicine
Bond University, Robina,
QLD 4229, Australia

2- Public Health and Neuroimmunology Research Unit
Faculty of Health Science and Medicine
Bond University, Robina,
QLD 4229, Australia.

Contact information: Sandra Ramos
Faculty of Health Sciences and Medicine
Bond University - Gold Coast
Queensland 4229, Australia
Phone 07 5595 1654
e-mail: sandra.ramos2@student.bond.edu.au

Abstract

It is reported that in both elite and recreational athletes Growth Hormone (GH) is the most commonly used drug aimed at improving sport performance. **Aim:** The purpose of the present study was to evaluate the immunomodulatory effects of short-term administration of recombinant human GH (rhGH) in healthy young males. NK cell number, activity and phenotype, T cell number, cytokine production of IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ and $CD4^+/CD8^+$ ratio with particular attention to the possible correlation to IGF-I production were investigated. **Methodology:** 30 healthy young males (27 ± 9 yrs) were randomly assigned to either placebo (n = 15) or drug (rhGH) 1mg/day groups (n = 15) with daily injection for seven days. IGF-I serum concentration and flow cytometry data were generated at baseline (day 0) and days 8, 15, 22 and 29 post injection. Data was analysed using a General Linear Model with repeated measures with a Bonferroni correction factor and significance set at $p \leq 0.05$. **Results:** The T cell cytokine secretion profile indicated a Th1 - Th2 shift. Interestingly, a significant difference ($p \leq 0.05$) of IL-10 mean fluorescence is noted from day 15 (P= 35.14; ± 19.93, rhGH = 26.63; ± 16.39) to day 22 (P= 61.32; ± 20.41, rhGH= 74.99; ± 46.91) and to day 29 (P= 101.98; ± 67.25, rhGH= 107.74; ± 122.58). Serum IGF-I concentration (ng/mL) increased significantly ($p \leq 0.01$) on day 8 (0.48 ± 0.78) after injections compared to baseline (0.31 ± 0.07) as well at day 15 (0.33 ± 0.06), 22 (0.29 ± 0.05) and 29 (0.29 ± 0.06). In conclusion, the cytokine secretion spectrum is affected by short-term rhGH administration in healthy young males being suggested to have induced $CD4^+$ T lymphocytes production of IL-10.

Key words: Exogenous human growth hormone; Cytokines; Killer cells, natural; Doping in sports, insulin-like growth factor I

1. Introduction

Several factors contribute to the reasons why both top level and amateur athletes use banned substances and include prospects of increased financial gain, the desire to become famous and a raising of social status (Aquino Neto, 2001). Despite the widespread belief that administration of growth hormone (GH) will enhance sport performance, there is little supportive data to match this belief. Although it is well known that GH induces hepatic IGF-1 secretion, little is known regarding its biological effects on immune function and effects on the sporting performance of healthy individuals. GH has been banned in sports competition and training and is classified as a prohibited substance within section S2 (Peptide hormones, growth factors and related substances) of the list generated by the world anti-doping agency (WADA, 2009).

Previous studies have shown that GH alters natural killer (NK) cell function. Interestingly, NK cells activity has been found to be significantly impaired in GH deficient (GHD) patients (Crist, Peake, Mackinnon, Sibbitt, & Kraner, 1987; Sneppen, Mersebach, Ullum, & Feldt-Rasmussen, 2002). An 18 month randomized, placebo-controlled, double-blinded trial study (n=110) showed that both NK cells total number, and subtype NK cells ($CD16^{+}$) were reduced in GHD patients compared to controls. (Sneppen, et al., 2002).

Peripheral blood NK cells can be subdivided into $CD56^{dim}$ $CD16^{+}$ and $CD56^{bright}$ $CD16^{-}$ NK cells (Caligiuri, 2008), which denote the normal and low cytotoxic activity respectively. Such phenotypic properties are responsible for unique functional attributes that directly impact on the human immune response (Cooper et al., 2001; Caligiuri 2008).

Other cells do express GH and IGF-I receptors and include B lymphocytes, macrophage, T lymphocytes and granulocytes precursors (Auernhammer & Strasburger, 1995). In GHD

patients suffering from hypopituitarism, they demonstrate immunological impairment parameters such as increased basal plasma levels of tumour necrosis factor-α (TNFα - 220%) and IL-6 by 340% (Serri et al., 1999). Other studies have shown that blood TNF-α levels are significantly higher in children with GHD than the controls, and long-term therapy with rhGH was effective at reducing such levels (Andiran & Yordam, 2007). *In vitro* administration of exogenous GH has been shown to improve a variety of immune cell functions including B lymphocyte antibody production and responses (Kimata & Yoshida, 1994), NK activity (Stephenson, Lee, Bailey, Shepherd, & Melling, 1991), macrophage activity (Gaytan, et al., 1994), T lymphocytes and neutrophil functions (Fu, et al., 1992). The $CD4^+$ T helper lymphocytes have a regulatory overall effect on immunity and orchestrate their activity through diverse cytokine secretions or by direct cell–cell contact. The $CD8^+$ cytotoxic T lymphocytes are effector cells that secrete and respond to cytokines that can target infected cells and induce programmed cell death (Kimata & Yoshida, 1994). The T cell immunological activity and response can be monitored by assessing the $CD4^+/CD8^+$ ratios that illustrate critical T cell functions (Kimata and Yoshida, 1994). Previous *in vitro* studies have shown that GH effects on normal and neoplastic human T cells enhanced proliferation is mediated by local increase expression of IGF-I (Geffner, et al., 1990; Merchav, Tatarsky, & Hochberg, 1988; Mercola, Cline, & Golde, 1981; Mosmann & Coffman, 1989).

The $CD4^+$ T lymphocytes demonstrate the production of a large number of cytokines known as Type 1 cytokines with IL2 and IFN-gamma being the main cytokines secreted. Enhanced activity of Th2 type cells evokes a strong antibody responses and relatively weak cellular activity (Cherwinski, Schumacher, Brown, & Mosmann, 1987; Mosmann & Coffman, 1989). Th2 cells produce cytokines known as Th2 cytokines and include IL4, IL6, IL10 and TNF-α. As previously mentioned, long-term therapy with rhGH administration in GHD children effectively reduces TNF- α level in these patients (Andiran & Yordam, 2007).

Importantly, IL-10 is well known for having essential immunoregulatory functions with direct influence on the activity of several leukocytes subsets. Its potent anti-inflammatory properties, suppress the expression of inflammatory cytokines such as TNF-α, IL-6 and IL-1 from activated pro-inflammatory macrophages (Donnelly et al., 1999). The IL10 induction of anti-inflammatory cytokines has not yet been explored in healthy individuals that have used rhGH.

As rhGH is currently used by elite and recreational athletes, it is important to further investigate the cellular effects of rhGH on the immune function of healthy males. The purposes of this study were therefore, to evaluate and monitor the immunological effects of seven days short-term administration of rhGH on: NK cell activity, number, phenotype subsets; the T cell numbers ($CD4^+$ and $CD8^+$) and production of cytokines IL2, IL4, IL6, IL10, TNF-α and IFN-γ: and finally the $CD4^+/CD8^+$ ratio.

Methods

Thirty healthy males were randomly assigned to either a treatment (rhGH, n=15, 24.5 ± 6.5 yrs) or Placebo (P, n=15; 26.1 ± 6.6 yrs) group. Daily injections of either rhGH (1mg) or placebo (0.9% sodium chloride) were administered intramuscularly during a period of seven days. Prior to each injection all participants received a standard breakfast (protein shake) followed by 15 min rest. Blood samples (EDTA and Heparin tubes) were collected via puncture of the antecubital vein by a qualified phlebotomist at baseline (day 0) and at days 8, 15, 22 and 29 after 7 consecutive days of injections.

All participants were required to read and sign an informed consent form that was approved by Bond University Human Research Committee. Exclusion criteria included: (i) regular use of therapeutic and/or recreational drugs, (ii) contraindications or risks to exercise (recent injuries/risk factors for maximal strength testing). Sports Medicine Australia pre-exercise

screening tool was used to filter out people at high risk for certain exercise related complications. The criterion for selection was based on the American College of Sports Medicine's (ACSM) guidelines (2000) for pre-exercise screening and testing.

Blood Analyses

The immunological analysis involved the assessment of NK cell numbers, function and phenotypes, $CD4^+/CD8^+$ numbers and further assessment examined cytokine production by Th1 and Th2 cells – (IL-2, IL-4, IL-6, IL-10, tumour necrosis factor (TNF)-α and interferon (IFN)-γ). All methods were performed using previously described methods (Marshall-Gradisnik, et al., 2008). Whole blood was collected into either heparinised or EDTA blood collection tubes and PBMC were isolated from using Ficoll–Hypaque density gradient centrifugation (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Determination of the lymphocyte phenotypic subsets ($CD4^+/CD8^+$) was performed using IMK lymphocyte test kit (Becton Dickinson Immunocytometry Systems, California, USA) as previously described (Marshall-Gradisnik, et al., 2008). List mode parameters were collected for 10,000 cells within the lymphocyte gate and positive staining was calculated based on the subsets control specimens. T cells were defined as $CD4^+CD3^+$T cells (T suppressor cells), $CD3^+CD8^+$ T cells (T helper cells), B cells as $CD19^+$B cells, and NK cells as $CD3^-$cells, ($CD16^+$and/or $CD56^+$). Flow-cytometry analyses were carried out using a FACSCalibur (Becton Dickinson) as described (Mozaffari, et al., 2004). Lymphocytes populations were identified by forward and side-scatter analyses.

The NK lymphocyte cytotoxicity was assessed as previously described (Marshall-Gradisnik, et al., 2008). Briefly, PBMCs were isolated from whole blood using ficoll-Hypaque (GE Healthcare) through density gradient centrifugation. The NK cells were labelled with 0.4% PKH-26 (Sigma, St Louis, MO) and resuspended at a final concentration of $5x10^6$cells/mL.

The target cells, K562 cell line were used at a concentration of $1x10^5$cells/mL. The K562 cells were cultured with NK cells in RPMI-1640 culture media (Invitrogen, Carlsbad, CA) for 4 hours in 37°C incubator with 5% CO_2, at a ratio of 25:1. Following incubation, cells were stained with Annexin V-FITC conjugated mAB and 7-AAD reagent (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. Percentage of cell death was measured via flow cytometry as previously described (Donnelly et al., 1999).

Quantification of NK cells phenotype and NK cells were isolated from whole blood according to manufacturer's instructions using RosetteSep Human Natural Killer cell Enrichment Cocktail (StemCell Technologies, Vancouver, BC) and Ficoll-Hypaque density centrifugation. Samples were then labelled with mAB CD56-FITC (BD Bioscience, San Jose, CA) and CD16-PE (BD Bioscience, San Jose, CA) according to manufacturer's specifications and analysed using flow cytometry.

The Th1 and Th2 profiles were assessed in whole blood samples 10mL of blood were collected into lithium heparin tubes from all participants (BD Bioscience) and layered over Ficoll-Hypaque for isolation of white blood cells (WBC) following centrifugation at 400g for 40 minutes and washed twice and resuspended in RPMI-1640 culture medium containing 5% foetal bovine serum (FBS). Isolated $CD4^+$T cells were mitogenically stimulated with or without 1μg of phytohemagluttinin (PHA). Cells were cultured in a 96 well plate at a concentration of $1x10^6$ cells/mL at 37^0C with 5% CO_2 for 72 hours. Supernatants were then removed from cells and assessed for Th1, Th2 cytokines using cytometric bead array kit (BD Bioscience) as specified by the manufacturer.

Double antibody radioimmunoassay system (RIA) was applied for quantitative *in vitro* diagnostic measurement of IGF-I within serum using a commercial RIA (IGF-I kit – Bioclone – Australia). Briefly, IGF-I was separated from its plasmatic binding protein then,

the analyte competes with ^{125}I labelled tracer antibody for binding to a constant amount of antibody. A second antibody coupled to magnetisable polystyrene particles was used to separate antibody bound from free ^{125}I labelled tracer antibody as specified by the manufacturer.

Data Analyses

The statistical analyses were performed using SPSS software (version 17.0). General Linear Model with repeated measures was applied and criterion for significant difference was set at $p \leq 0.05$ with Bonferroni post hoc correction applied.

Results

Both groups were not significantly different ($p>0.05$) in height (P; 178.9 ± 5.9 cm, rhGH; 178.8 ± 9.5 cm), weight (P; 83.1±9.1 kg, rhGH; 81.2±17.9 kg) or age (P; n=15; 26.1± 6.6 yrs; rhGH 1mg/day; n=15; 24.5 ± 6.5 yrs).

The flow cytometry results revealed no significant differences ($p>0.05$) between or within groups for NK cell numbers, phenotype or NK activity from baseline (day 0) to day 29 (Table. 1).

Table 1. Natural killer cells absolute numbers (Mean ± SEM), lysis and phenotype in MF(AU) from day 0 to day 29 for P (placebo) and rhGH groups.

Assay	Group	Day0	Day8	Day15	Day22	Day29
NK number	P	669±325.1	587.8±222	611.4±322.8	555.5±348.3	550.7±270.4
	rhGH	560.4±328	492.5±229.7	492.8±190.3	474.79.±160.9	535.2±154
Nk lysis	P	35.30±4.57	41.96±6.58	67.80±3.87	31.42±4.76	40.80±6.61
	rhGH	34.85±7.63	33.91±5.97	61.64±3.17	41.18±5.73	44.23±7.23
Nk phenotype	P Bright	217.8±162	224.8±67	257±72	197±161	203.3±104
	rhGH Bright	313.8±158	402.3±345	302.1±221	281.3±86	242.1±140
	P Dim	5.606±2011	5.428±1091	5.788±866	4.391±2653	4.860±1429
	rhGH Dim	5.687±1245	5.309±1827	5.072±2032	4.937±1175	5.210±1034

The T cells number ($CD4^+$ and $CD8^+$), $CD4^+/CD8^+$ ratio and cytokyne production by TH1 and TH2 were also analysed by flow cytometry and showed no significant differences between or within groups for $CD4^+$ and $CD8^+$ numbers from Day 0 (P= 3700.08±315.52 mean fluorescence) or for $CD4^+/CD8^+$ ratio from day 0 (rhGH=3751.00±297.7 and P=1.35±0.40; rhGH=1.26±0.43) to Day 29 (P=3765.42±293.5; rhGH=3830.2±149.63 and P= 1.55±0.47; rhGH=1.28±0.44 mean channel fluorescence).

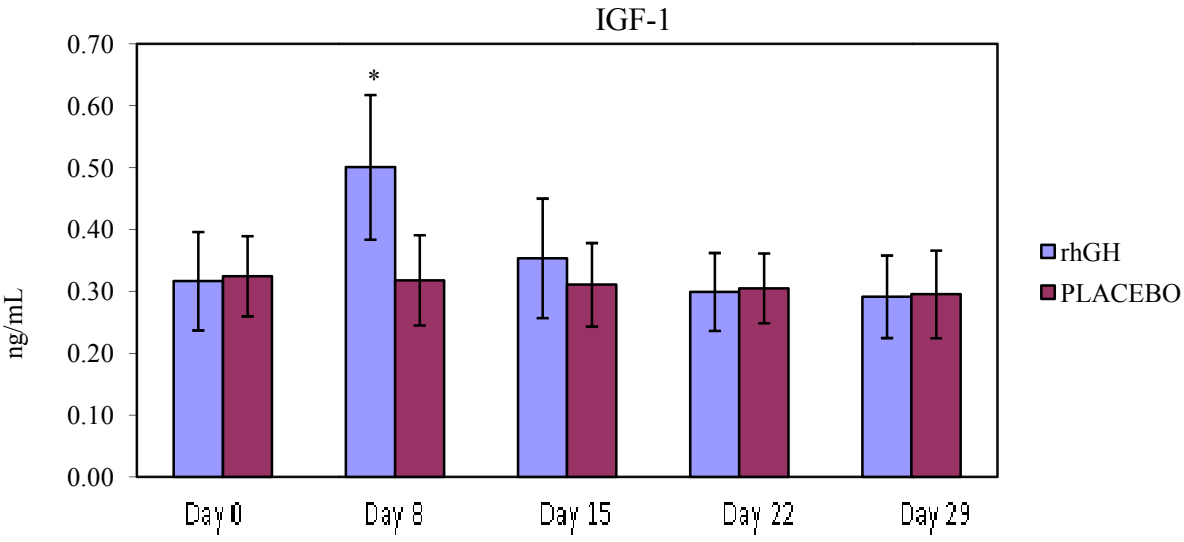
The T cell cytokyne production of IL10 increased significantly in rhGH group from day 15 (P=46.32±44.16; rhGH=30.16±23.09 mean channel fluorescence) to day 22 (P=74.28±36.10; rhGH=65.32 ± 35.37 mean channel fluorescence) and remained increased at day 29 (P= 107.50±60.92; rhGH=57.21±54.57 mean fluorescence) as shown in Figure 2. In contrast, IL2,

IL4, IL6, TNF-α and IFN-γ showed no significant ($p \geq 0.05$) difference from baseline to day 29 in both groups (Table 2.).

Table 2. T cell cytokine production from baseline to day 29 for placebo (P) and rhGH groups in MF(AU).

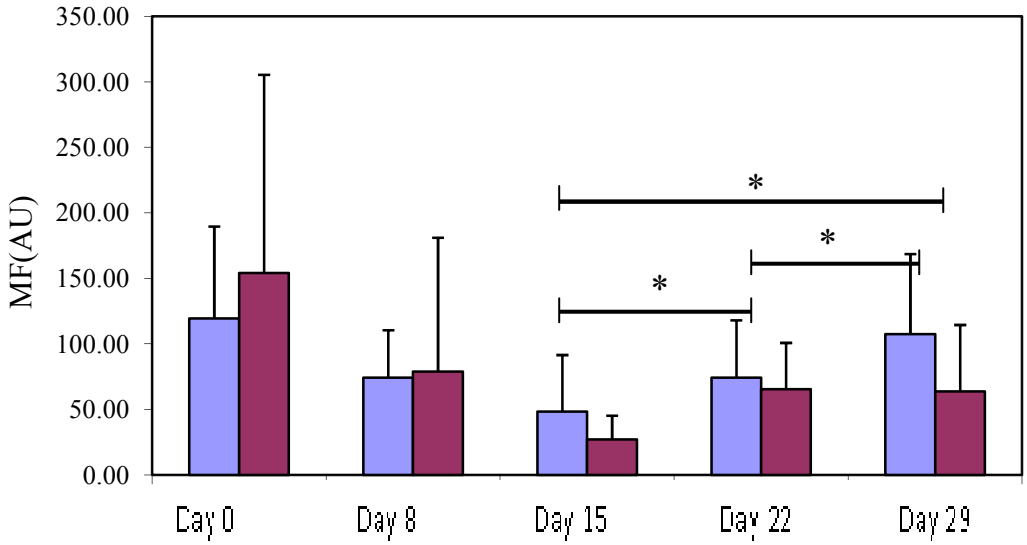
Assay	Group	Day0	Day8	Day15	Day22	Day29
IL2	P	427.77±195.76	501.30±201.52	374.60±144.90	401.76±192.02	519.94±181.65
	rhGH	517.02±115.57	345.29±172.65	355.30±202.78	464.27±217.19	499.13±153.21
IL4	P	8.29±3.22	7.47±2.36	8.93±3.79	8.05±1.76	12.42±5.97
	rhGH	10.08±3.83	7.08±2.20	8.67±6.39	8.60±5.65	13.69±9.67
IL6	P	4796±1608	4193±1814	2635±1666	2188±1733	3730±1941
	rhGH	5289±1862	3243±1512	3122±1478	3670±2025	3584±1651
TNF-α	P	156.70±60.78	138.33±37.80	135.90±96.87	102.65±42.18	125.73±52.39
	rhGH	141.81±46.94	180.33±73.80	131.64±59.42	152.22±72.23	131.02±54.61
IFN-γ	P	481.73±175.98	373.19±213.82	368.45±250.25	338.02±150.64	430.83±254.47
	rhGH	453.15±258.86	382.38±236.18	382.77±155.94	294.37±161.92	296.51±161.27

A significant difference ($p \leq 0.01$) was found in serum IGF-I concentration in the rhGH group at day 8 (0.49 nmol/mL) compared to baseline (0.32 nmol/mL) (Figure 1.) while no other time point was significant for this group. There was no significant difference in serum IGF-I for the control group from baseline to day 29.



* Significant difference between day 8 and days 0, 15, 22 and 29 for p≤0.05 in the rhGH group.

Figure 1. IGF-1 analysis in serum per collection day (0 to 29) for groups.



* Significant increase (p≤0.05) from day 15 to days 22 and 29 in IL10 secretion by the rhGH group.

Figure 2. IL10 secretion from T cells from baseline to day 29 of the study.

Discussion

The aims of this study were to evaluate in young males, the immunomodulatory effects of short-term administration of rhGH on NK cell number, activity and phenotype, T cell cytokine production of IL2, IL4, IL6, IL10, TNF-α and IFN-γ and $CD4^+/CD8^+$ ratio in human PBMCs, with particular attention to the possible correlation to IGF-I production.

The present study indicated no changes in NK cell count, phenotype or activity in either the experimental or placebo group. In contrast, in experiments with 24 month old Wistar rats, rhGH replacement therapy (2 mg/kg daily for 10 weeks) significantly improved several lymphocyte functions such as NK cell activity (Baeza, et al., 2008). Interestingly, a previous study confirmed that both total NK cell count and particularly $CD16^+$ NK cell subtype were reduced in GHD patients compared to controls (Sneppen et al., 2002). However, the studies presenting significant results on NK cell were performed for a period of at least ten weeks, suggesting the time course of GH replacement may be a key factor.

In addition, serum IGF-I level significantly increased on day 8 within the rhGH group and along with the high numbers of IGF-I receptors known to be on NK cells (Kooijman, et al., 1992), it was predicted that NK cells activity would be altered after short-term administration of rhGH. However, rhGH induce IGF-I release did not alter NK cells activity nor their number or phenotype in our study. Serum IGF-I concentration in the rhGH group and Placebo group showed only a weak correlation with NK cell activity.

The current results indicate that IGF-I has the potential to exert anti-inflammatory actions through stimulation of IL10 production in $CD4^+$ T lymphocytes. The IL-10 cells may inhibit the production of Th1 cytokines (Moore, de Waal Malefyt, Coffman, & O'Garra, 2001) leading to the prevalence of Th2 cells. IGF-I may induce a shift from the production of Th1 to Th2 cytokines, leading to a diminution of cellular immune responses and a stimulation of

antibody-mediated responses. It would appear that rhGH for short-period of time may exert its stimulation effects of Th2 through IL 10 release in healthy population with possible anti-inflammatory action.

The rhGH therapy is a recognised procedure for enhancing immune function in GH deficient populations (Crist et al., 1987; Sneppen et al., 2002; Serri et al., 1999; Andiran and Yordam, 2007), and our data suggest that a dose of 1mg/day for seven days may potentiate immune function of healthy young males through the significant increase in the release of IL10 by $CD4^+$ T lymphocytes.

Conclusion

The rhGH has been used by athletes with the intention of improvement of performance. Moreover, there is no evidence of its effect on the immune function. In this paper, it was shown that rhGH stimulated IL10 secretion by $CD4^+$ T cells in healthy young males.

Although the lymphocyte numbers between both groups were not significantly different from baseline to day 29, cytokine production showed a non significant decrease followed by a significant increase from day 15 to day 22 and 29 and from day 22 to day 29. In conclusion, cytokine release can be affected by short-term rhGH administration in healthy young males suggesting that rhGH may act on $CD4^+$T lymphocytes by increasing IL10 production and may then be a key component in T cell activation.

Conflict of interest statement:

The authors have no conflicts of interest regarding this research.

References

See page 132.

Study Three

Assessment of gene expression in healthy young males after administration of short-term recombinant human growth hormone.

S. Ramos[1], E. Brenu[1], F.R.A, Neto[2], B. Gray[1], M., Van Driel[1], L.R. McNaughton[1], L. Tajouri[1], S. Marshall-Gradisnik[1]

1 – Faculty of Health Science and Medicine

Bond University, Gold Coast

Queensland 4229, Australia

2 - LABDOP – LADETEC

Federal University of Rio de Janeiro, Chemistry Institute

Rio de Janeiro, Brazil

CEP 21941-909

Contact information: Assistant Professor Lotti Tajouri

Faculty of Health Sciences and Medicine

Bond University, Gold Coast

Queensland 4229, Australia

Ph: +61 7 55951148

Email: ltajouri@bond.edu.au

Abstract

Introduction: Limited studies have provided little evidence for the effects of exogenous recombinant human growth hormone (rhGH) on the immune response at and through the gene level. The aim of this study was to evaluate the effects on the gene expression of Interleukin 6 (*IL6)*, Interleukin 6 receptor (*IL6R)*, Interleukin 10 receptor (*IL10R)*, Perforin (*PFR1)*, Nuclear Factor Kappa B (*NFKB)* and Granzyme A (*GZMA)*, genes related to immune function and regulation, after short-term administration of rhGH. **Methodology:** Thirty males (age: 27 ± 9 yrs) were randomly assigned to either placebo (P) or rhGH (1mg/day for seven days) groups. Gene expression was evaluated by microarray and confirmed by quantitative real time PCR (q-RT PCR) analysis for both groups following P and rhGH groups pooling of cDNA. Data was generated at baseline (day 0) and days 8, 15, 22 and 29 post injections. **Results:** The ratio of expression at each time point post baseline was normalised against baseline expression values. On day 8 all evaluated genes except *IL6* presented an up-regulation in fold of expression in the ratio against baseline in the rhGH group in contrast with the placebo group. From day 8 to day 29 all the evaluated genes apart from *IL6* remained up-regulated in relation to baseline in the rhGH group. **Conclusion:** Short-term administration of rhGH was effective in elevating the gene expression of *IL10R, IL6R, NFkB, GZMA and PFR1* suggesting the activation of anti-inflammatory responses.

Key Words: Gene expression, recombinant human growth hormone, immune function, q-RT PCR.

Introduction

A number of neuroendocrine hormones produced in the anterior pituitary, for example growth hormone (GH), have particularly prominent effects on immunity which have been shown to exert stimulatory property on both human and murine T cells *in vitro*, including enhancement of T cell proliferation and functional antigen responses [1, 2]. Human GH regulates various components of the immune system, such as lymphopoiesis [3] and immunological cytokine mechanisms [4].

The physiologic functions of GH are mediated by two different but complementary mechanisms: it directly activates target organs via specific GH receptors while indirectly its effects are mediated through insulin like growth factor 1 (IGF-I). Insulin like growth factor 1 and its receptor (IGF-IR) provide a potent proliferative signalling system that stimulates growth in many different cell types and blocks apoptosis. *In vivo*, IGF-1 acts as an intermediate of many growth hormone responses [5].

The anabolic actions of human GH have made it attractive as a potential ergogenic agent for athletes although there is a lack of evidence to support increased performance in sports after rhGH use. However, it is highly plausible and likely, that elite athletes are using rhGH in dosages ranging from approximately 15 to 180μg/kg per day, which would be significantly higher than dosages used in most experimental studies [6].

In vivo studies have shown that GH administration increases natural killer cell (NK) activity [7]. Furthermore, GH increases cytokine transcript levels in lymphoid cells in vitro [8] and IGF-I generated, in response to GH, can act on immune cells giving rise to a secondary response [3].

Several authors [9-11] have recently explored the use of peripheral blood mononuclear cells (PBMCs) as a source of material for gene expression analyses in humans. This current study

used PBMCs to evaluate the effects of rhGH administration in the gene expression of selected genes with influence on the immune function.

Growth hormone signals through the GH receptor (GHR), which couples to the cytoplasmic tyrosine kinase, Janus kinase 2 (JAK2) [12], and GH has been shown to activate STATs 1, 3, and 5 [13]. The STAT proteins play a prominent role in the GH-induced regulation of gene transcription [14]. RT-PCR is a highly sensitive and versatile technique for amplification and quantification of RNA [15].

The aim of this study was therefore to determine if selected genes related to the immune function and regulation (Interleukin-6 (IL6), Interleukin-6 receptor (IL6R), Interleukin-10 receptor (IL10R), Perforin-1 (PFR1), nuclear factor κB (NFκB) and Granzyme A (GZMA)), important to the inflammatory and anti-inflammatory responses are influenced by short-term administration of rhGH.

Methods

Thirty healthy young males were recruited and randomly divided into two groups: rhGH (n=15, 24.5 ± 6.5 yr) for the treatment group and placebo (n=15; 26.1 ± 6.6 yr). Volunteers were excluded on the basis of regular use of therapeutic and/or recreational drugs and contraindications or risk to exercise (recent injuries/risk factors for maximal strength testing). Daily injections of either rhGH (1mg Genotropin "Miniquick", Pfizer, Sydney) or placebo (0.9% sodium chloride) were intramuscularly administrated for seven days. Prior to each injection all subjects received a standard breakfast (protein shake, Body Science, Australia).

Subsequent to the injections, collected blood samples from all participants were assessed for gene expression at the following times: prior to (day 0) injection and seven (day 8), 15 (day 15), 22 (day 22) and 29 (day 29) days after the injection. Subjects were required to read and sign an informed consent form prior to the experiment and the study was approved by Bond University Human Research Ethics Committee.

Blood collection & white blood cell extraction

Blood samples from subjects were collected into 4mL EDTA blood tubes. Peripheral blood mononuclear cells extraction was carried out in Ficoll-paque and the protocol consisted of the following: within a 14 mL Falcon tube, 4 mL GIbco PBS, pH 7.4 sterile was added to 4 mL of fresh blood; Tubes were then inverted 3 times and 8 mL of diluted blood was layered over 4 mL of Ficoll-paque. Post centrifugation at 900 RCF for 20 min/room temperature, plasma was removed gently (stored at -80C°) and WBC were collected using with a 1000µL pipette (filter tipped) and spun down at 4C°/4000 RCF/10 min and rinsed in PBS, pH 7.4 and finally stored at -80C° for further processing.

Extraction of total RNA

Total RNA was extracted from PBMC stabilized in RNA tubes, amplified, and hybridized to Illumina HumanRef-8v2 bead chips each representing >24,000 sequences representing NCBI RefSeq genes.

The TRizol® Plus RNA purification kit (Invitrogen Australia Pty Limited) was used to isolate total RNA. Briefly, sample tubes were removed from a -80°C freezer and placed at room temperature with TRizol® Plus RNA purification kit (Invitrogen). Lysis of cells was performed by passage through pipette tips several times and incubated for 5 min at room temperature to allow complete dissociation of nucleoprotein complexes. 0.2 mL chloroform was then added and gently mixed by hand for 15 sec. Following 3 min incubation (room temperature), the sample was centrifuged at 12,000 x g for 15 min at 4°C. A colourless upper phase containing the RNA was collected gently and transferred into a new tube containing an equal volume of 70% ethanol. Samples were vortexed for 30 sec and placed above RNA Spin cartridge. Binding of RNA, washing steps and elution series were undertaken following manufacturer's instructions. The RNA was eluted into 100 µL of pure water and quality control was established on RNA samples. Assessment of both RNA concentration and purity

was executed with the NanoDrop 1000 Spectrophotometer. RNA quality was assessed by taking into account ratio measurements for both 260/280 and 260/280 ratios.

Microarray and cDNA synthesis:

In this study, we analysed gene expression profiles of individuals prior to injection (baseline) and following a sequence of doses of 1mg recombinant GH administered intravenously daily for seven days. 0.9% sodium chloride was administered daily to the Placebo group for seven days. Blood samples were collected at baseline and in weekly intervals post injection (day 8 – time point 1) and following times (day 15, day 22 and day 29 corresponding to time points 2, 3 and 4 respectively). RNA extraction from whole blood PBMCs was followed by microarray experiments using the Illumina chip platforms. Cluster analysis in this study revealed a collection of genes with altered expression.

The synthesis of cDNA was undertaken using the SuperScriptTM III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen Australia Pty Limited). Briefly the methods consisted of making a reaction mix [10µL of 2X RT Reaction Mix; 2µL of Reverse Transcriptase Enzyme Mix; 8µL of Eluted RNA (2 µg)], observing incubation a 10 min at 25°C, followed by a second incubation at 50°C for 30 min. The reaction was stopped by bringing the reaction at 85°C for 5 minutes. The overall cDNA was stored at -20°C.

Following collection, cDNA from control samples was pooled at equal amounts and cDNA from samples were pooled in the same manner. 4 µL of diluted cDNA (1 in 100 dilutions) was used as templates. Sybr mix including Taq polymerase (iQ^{TM} SYBR$^{®}$ Green Supermix; BIO-RAD - USA) as well as forward and reverse primers (0.5 µM each; see Table 1) (Geneworks, Australia) were added to the cDNA for a final volume of 25 µL. Primers were previously designed using primer 3 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

Table 1. PCR primers designed for detecting specific genes related to the immune function. All primer sequences are presented in 5' to 3' orientation.

Gene name	Genes	Unigene accession number	Forward	Reverse
Granzyme A	GZMA	Hs.90708	CTGTGATTGGAATGAATATGGTTTGT	CAGAATCTCCATTGCACGAGTCT
Interleukin 6 receptor	IL6R	Hs.709210	CACGACTCTGGAAACTATTCATGCTA	GGGAACATCCACCAGCAAGT
Interleukin 6	IL6	Hs.654458	AAGCAAGTGGGATCACCTATGAA	GCAATGTCTTCCACACGAGTTG
Interleukin 10 receptor	IL10R	Hs.504035	TCAGTCACTTCCGAGAGTATGAGATT	CTTTCTTGTGTGTGAACGTGAAGTTT
Perforin 1	PFR1	Hs.2200	CCGCTTCTACAGTTTCCA	GTAGTTGGAGATAAGCCTGAG
Nuclear Factor Kappa B	NFKB	Hs.618430	GGAAGTACAGGTCCAGGGTATAGCT	CATGCTTCATCCCAGCATTAGA
Ribosomal RNA 18s	18s		TTCGAGGCCCTGTAATTGGA	GCAGCAACTTTAATATACGCTATTGG

Detection of PCR product in real time was performed using the Corbett Research Rotor-Gene 3000. The PCR volumes were placed in individual Corbett 0.1 mL strip tubes on -20°C pre-cool metal tube carriers with this order: cDNA and PCR mix. Tubes were closed off and manually handled to allow the whole PCR solution to be at the bottom of the tube. The following cycling conditions were used and entered in the Rotor Gene software (Cycle 1, 94^{o}C 4 minutes (x1), Cycle 2, 94^{o}C 30 s, 59^{o}C 1 min, 72^{o}C 30 sec (x45)). A post-PCR amplification protocol was preset before the run to obtain melt curve representations (ramping from 50 to 99°C with 1°C ramping every 5 seconds).

Data Analysis

cDNA was synthesised from RNA extracted from human peripheral blood mononuclear cells. 18s was used as an internal standard house keeping that reflects accurately mRNA expression levels in PBMCs [16]. Following q-RT PCR analysis, quantification cycle (Cq) values from triplicates were collected at linearity and used to calculate the 18S rRNA corrected Cq (or ΔCq) for each gene. Triplicate repeat ΔCq were assigned with ± SE determinations and were then used to calculate the mean corrected difference in Cq for placebo and rhGH (or ΔΔCq). The extent of the response was determined by 2-mean (ΔΔCq) (formula which indicates the copy number) while a negative value suggests repression of receptor expression, so the relative degree of response is calculated by 2-mean (ΔΔCq). The absolute fold change is used to denote changes in expression; i.e. a 2-fold increase is equivalent to a doubling of expression. cDNA pools of placebo and rhGH samples were used as PCR templates for RT PCR experiments to determine relative degree of responses. Our placebo pooling approach, with equivalent power, has been previously published in the literature [17, 18]. The mean ΔCq value was calculated for both the gene and the internal control. Cq values were determined using a threshold line that was set at the exponential phase of all PCR runs and therefore calculated for optimum PCR efficiency. All ΔCqs were then consequently used to

calculate 2-ΔΔCq which represents the relative expression levels and allows comparison between baseline and time-points. Each RT-PCR was run in triplicate with standard deviation less than 0.2.

Results

The efficiency of all PCR ranged from 90 to110% meaning doubling of the amplicon at each cycle (PCR slopes ranging from -3.1 to -3.6). Therefore the basis 2 is expected and does not mirror the real PCR efficiency. The q-RT PCR results indicate rhGH and placebo groups presented similar expression for the evaluated genes at baseline. On day 8, all of the evaluated genes other than IL6 presented an up-regulation in the ratio against baseline in the rhGH group (see Table 2).

Table 2. Normalised ratios of expression in immune selected genes on day 8, day 15, day 22 and day 29 relative to the baseline expression of rhGH group compared to Placebo group. The relative degree of response (expressed as ratios) was calculated by 2-mean (ΔΔCq) and denote changes in gene expression.

Genes	Day 8	Day 15	Day 22	Day 29
NFκB	1.18	4.44	2.77	2.33
IL6	0.52	0.58	0.59	0.17
PFR	2.18	2.11	2.14	3.22
IL10R	2.27	3.68	3.19	4.57
IL6R	1.77	2.41	1.57	1.8
GZMA	2.16	4.96	2.11	2.28

Interestingly, on day 15 with the exception of *IL6* (0.50 fold), all evaluated genes in the rhGH group presented an up-regulation above two fold compared to the placebo group; *IL6R* – 2.41, *PFR1* – 2.11, *IL10R* – 3.68, *NFκB* – 4.44, *GZMA* - 4.96 fold.

Although *NFκB* had a significant drop from day 15 (4.44 fold) to day 22 (2.77 fold), its expression remained up-regulated in the rhGH group compared to placebo in relation to baseline. *PFR1* presented very similar results from day 15 to day 22 with an up-regulation of

2.14 fold in the rhGH group compared to the placebo group. A significant decrease was shown for genes *GZMA* and *IL6R* from day 15 to day 22 (4.96 to 2.11 fold and 2.41 to 1.57 fold respectively). Interestingly, *IL10R* presented a non-significant drop from day 15 (3.68 fold) to day 22 (3.19 fold) following a 4.57 fold increase in up-regulation on day 29 in the rhGH group compared to the placebo group in relation to baseline.

On day 29 *GZMA* fold expression increased compared to day 22 in the rhGH group, remaining up-regulated (2.28 fold) compared to the placebo group. The *PRF1* on rhGH group reported a significant increase in its expression on day 29 (3.22 fold) compared to day 22 and compared to the expression in the placebo group relative to baseline.

There was no significant difference in the *IL6* gene expression between or within groups from day 0 to day 29 of the study (Table 2).

Discussion

Our study shows that short-term administration of rhGH has an effect on the pro- and anti-inflammatory gene expression regulation by significantly increasing the expression of *IL10R, NFkB, GZMA and PFR1*.

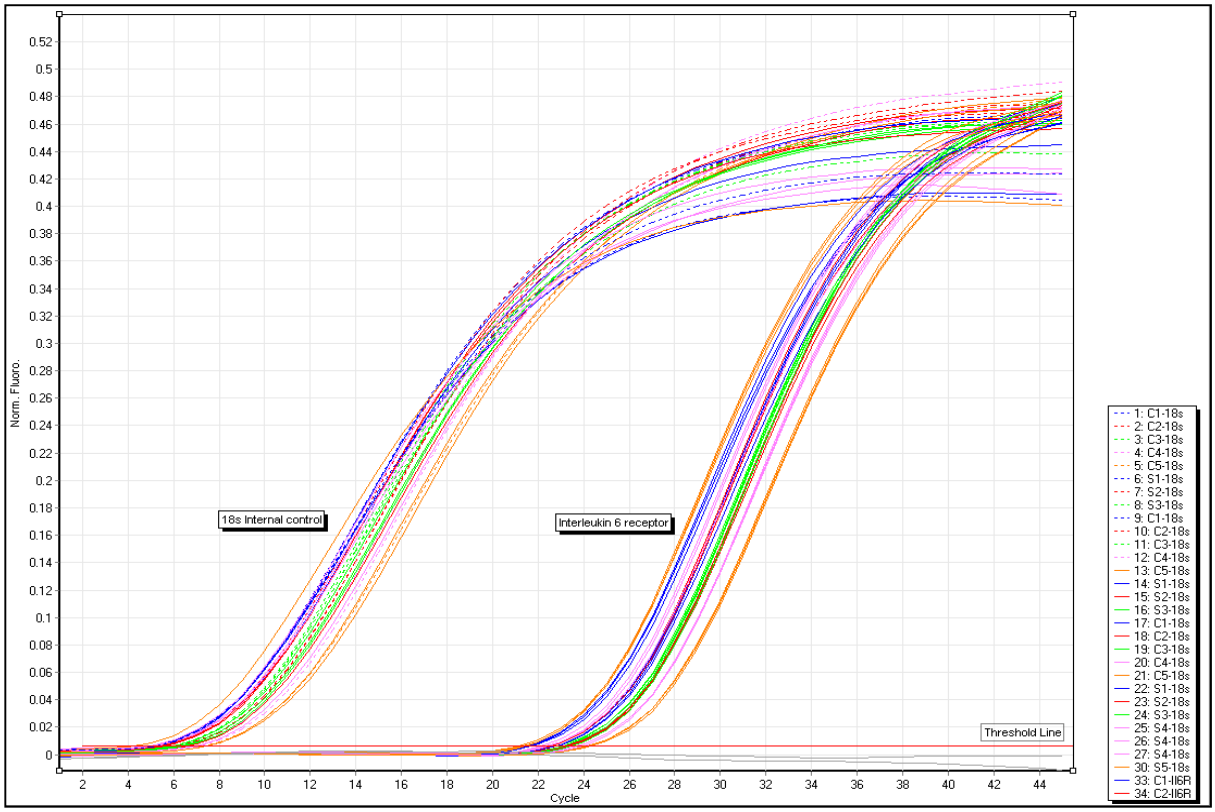
The granzyme A and *PFR1* presented a similar pattern of up-regulation associated to *NFκB* up-regulation, once more confirming a profile of increased anti-inflammatory responses in the rhGH group compared to placebo. A previous study [19] evaluated the effects of exercise (30 min of constant work rate cycle ergometry (80% peak O_2 uptake)) on the gene expression, measured using microarray analysis. The GZMA gene expression reported a 2.0 fold increase from before to immediately after the exercise bout. However, from the end of exercise to after 60 minutes of recovery a down-regulation to -2.97 fold change was observed. After 60 minutes into recovery, samples showed down-regulated compared to before exercise samples as well. According to our results, rhGH increased anti-inflammatory

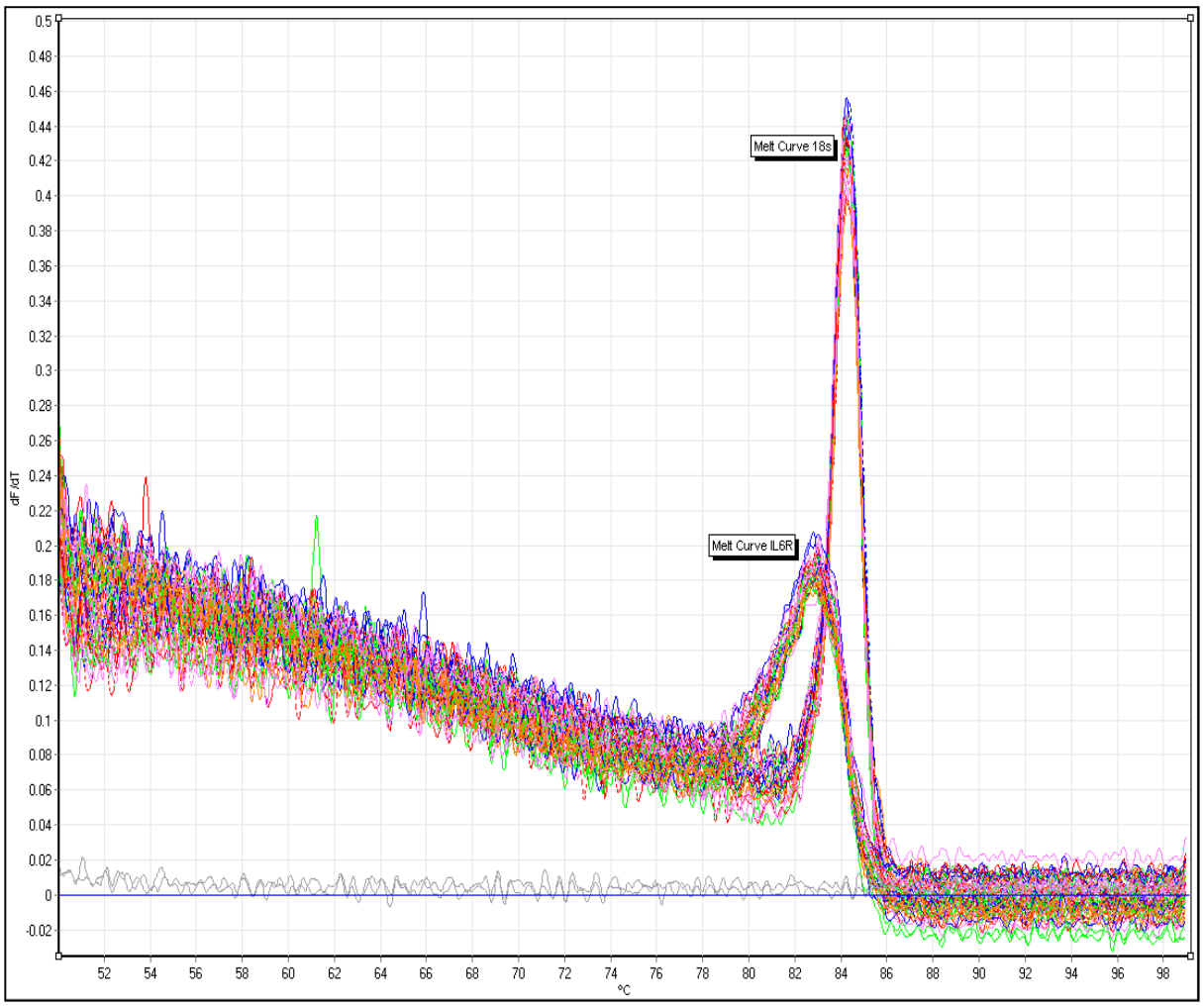
activation through GZMA, PRF1 and NFkB mRNA up-regulation in healthy young males, suggesting an advantage to athletes and their stressed immune system.

As an effect of rhGH administration the pro-inflammatory genes such as *IL6* would be inhibited with the *NFκB, GZMA, PRF1, IL10R* gene expression up-regulated. Miles and col. [20] evaluated the perforin gene expression using quantitative RT–PCR from moderately trained runners (males from 18 to 40 years old) and compared it to controls before exercise, immediately after (0h), 1.5, 5, and 24 h following a 60 minutes run at 80% of VO_2 peak. The exercise bout decreased the perforin gene expression in the runners group from before to after exercise and this decrease was maintained over 24 h. Once again the effects of rhGH administration suggest a possible benefit to athletes by up-regulating *NFκB, GZMA, PRF1* mRNA.

In our study no difference in the *IL6* gene expression was noticed between or within groups from day 0 to day 29 of the study (Figure 1).

Figure 1. qRT PCR (top) and melt curve analysis (bottom) results of *IL6R* gene and *18s* gene as the keeping house gene.


Norm. Fluoro.
Cycle
18s Internal control
Interleukin 6 receptor
Threshold Line
1: C1-18s
2: C2-18s
3: C3-18s
4: C4-18s
5: C5-18s
6: S1-18s
7: S2-18s
8: S3-18s
9: C1-18s
10: C2-18s
11: C3-18s
12: C4-18s
13: C5-18s
14: S1-18s
15: S2-18s
16: S3-18s
17: C1-18s
18: C2-18s
19: C3-18s
20: C4-18s
21: C5-18s
22: S1-18s
23: S2-18s
24: S3-18s
25: S4-18s
26: S4-18s
27: S4-18s
30: S5-18s
33: C1-IL6R
34: C2-IL6R


dF /dT
°C
Melt Curve 18s
Melt Curve IL6R

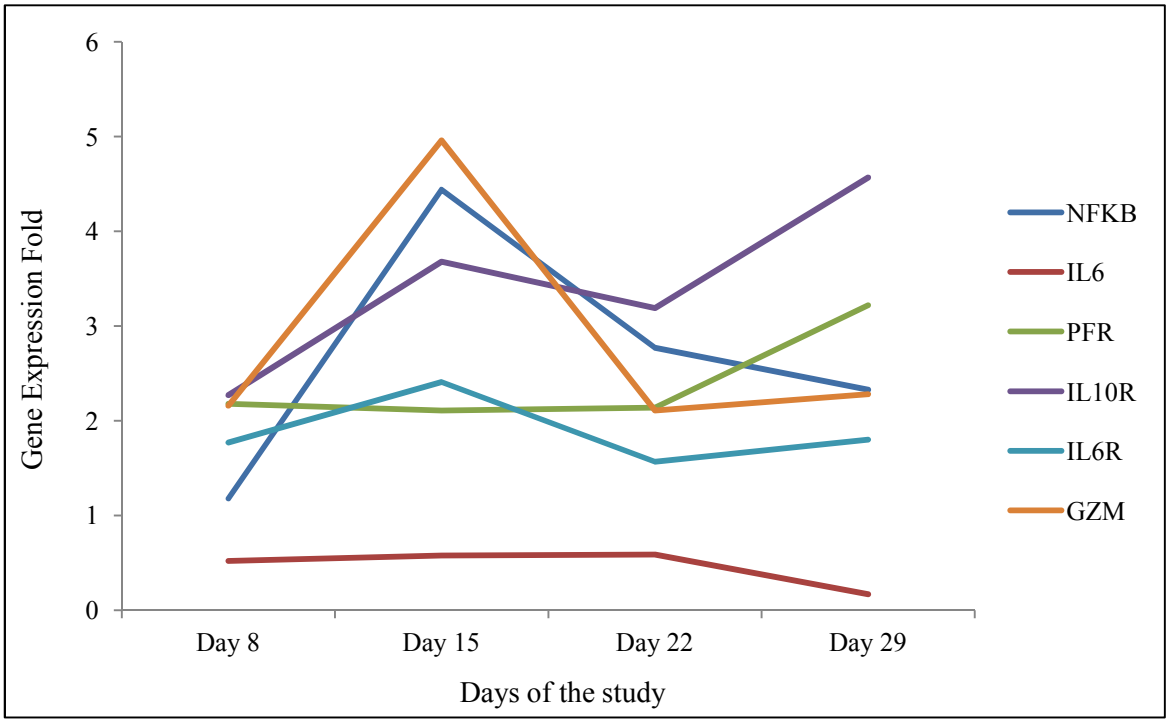
In contrast, *IL6R* showed increased expression from day 0 to day 29 with little variation along the evaluated days.

Growth hormone therapy over a period of 12 weeks in a randomized crossover comparison was previously shown [21] to mediate in the various stages of inflammatory processes in patients with idiopathic dilated cardiomyopathy by significantly reducing ($p \leq 0.04$) pro-inflammatory cytokines such as IL6, as well as significantly increasing the ratio IL10/IL6.

In athletes, the effects of ultra distance (245 km) run considerably increased plasma IL-6 levels by 8000-fold (7781.0 ± 8317.3 pg/ml) immediately after (within 15 minutes) the end of the race, returning to baseline level 48 hrs after the race (0.7 ± 0.5 pg/ml) [22].

Interleukin 6 transmits its biological signal through IL6 receptor, an IL6 specific binding molecule. Although IL6R mRNA increased in the rhGH group after administration of rhGH, IL6 presented unaltered expression. The ligand of IL10R, IL10 has potent anti-inflammatory properties inducing the repression of the expression of inflammatory cytokines such as IL6 by activated macrophages [23]. In our study the up-regulation of IL10R above 2 fold with increases along the evaluated days until the end of the study suggests an effect on the inhibition of IL6 along the evaluated days. The regulation of IL6 also depends on the protein transcription factor NFκB which in our study showed a 4.44 fold increase in its expression (See Figure 2).

Figure 2. Selected genes expression of rhGH group compared to Placebo group from day 0 to day 29 of the study in relation to baseline.



According to some authors [24] NFκB has a role in the recovery from exercise which is the induction of pro- inflammatory genes to facilitate post-exercise regenerative responses in damaged tissue.

This could indicate that beside the unaltered expression of IL6, up-regulation of IL6R could potentially trigger more intracellular cascades, including NFκB activation.

As described by Schottelius and col. [25], in the nucleus NFκB binds to target DNA elements and regulates the transcription of genes involved in immune and inflammatory responses.

The NFκB transcription pathway was shown previously to be a key regulator of IL6 cytokines [26, 27]. The NFκB has been cited to coordinate the activation of several genes in response to pathogens and pro-inflammatory cytokines that are considered crucial in the activation of acute as well as chronic inflammatory diseases [25].

Both receptor genes evaluated in our study, IL6R and IL10R, presented a consistent increase in their expression following the rhGH administration, and as previously reported by Lai and col. [28] IL10R is highly effective in recruiting the signalling pathways of IL6 cytokine

receptors and mediating the immunosuppressive signal of IL10, and thus it inhibits the synthesis of pro-inflammatory cytokines.

In a recent study that applied flow cytometric analysis, Ramos et al. [29], demonstrated a time effect of exogenous rhGH on the IL10 production by T helper cells. In this study, IL10 was shown to be increased in the rhGH group compared to the placebo group 16 days after the last day of 1mg of exogenous rhGH. The anti-inflammatory action of IL10 in T cells of healthy young males may enhance the cascade activation mediated by IL10R.

In conclusion, short-term administration of rhGH was effective in up-regulating the gene expression of IL10R, IL6R, NFkB, GZMA and PFR1, genes normally affected by exercise and important for the immune function regulation suggesting the activation of anti-inflammatory responses by rhGH.

Conflict of interest statement

The authors have no conflicts of interest regarding this research

Reference

See page 132.

Chapter 4

Discussion

4.0 Discussion

This is the first study to administer rhGH to healthy young males aiming to evaluate the possible effects on their blood rheology, immune system and gene expression. The variables chosen are fundamental influences on sports performance as well as important indicators of health status. We demonstrated that 7 days of rhGH (1mg) administration in healthy young males induced significant alterations in the blood rheology, specifically an increase in the native aggregation of RBC. We have also shown that rhGH may perhaps stimulate a chronic IL10 secretion by $CD4^+$ T cells, while T lymphocyte numbers and percentage remained the same throughout the study. We have demonstrated up-regulation of mRNA expression of *IL10R*, *IL6R*, *NFκB*, *PFR1* and *GZMA* genes.

4.1 Blood rheology aspects

Alterations in blood rheology, especially increased erythrocyte aggregation, play an important role in the development of arterial and venous thrombotic lesions (Demiroglu et al., 1998). Erythrocyte aggregation is one of the important hemorheological determinants that may create problems at the level of microcirculation (Puniyani et al., 1991). It is stated that erythrocyte aggregation has a direct effect on the formation of thrombi at low shear conditions (Stoltz and Donner, 1987). However, the results for the native aggregation index showed only an effect immediately following administration of rhGH but not on days 15, 22 or 29 of the study.

Although aggregation of erythrocytes was immediately increased in our study, plasma fibrinogen was unaltered from day 0 to day 29 in both groups suggesting that rhGH has no influence on fibrinogen. Additionally, fibrinogen cannot be considered a factor in the observed increase of erythrocyte aggregation. Fibrinogen was previously demonstrated by Miljic and colleagues (2006) to be unaltered in adults with growth hormone deficiency

(GHD), remaining constant after daily GH replacement for 12 months. However, Jeejeebhoy al., 1970 in a landmark study, administered bovine growth hormone to rats and observed within a few hours a significant increase in the synthesis of fibrinogen. Perhaps the methodological differences within doses, collection times and samples between studies lead to the different results.The deformability of erythrocytes remained the same within and between groups from the beginning until the end of our study which indicates preservation of the membrane of the red blood cell after rhGH administration.

The full blood count indices such as total red blood cells, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), platelets and platelets mean volume (PMV) showed no difference between groups before or after administration of rhGH. Peyreigne et al., (1999) evaluated the main blood rheology parameters in adults with growth hormone deficiency (GHD). They found no difference in the blood rheology between controls, matched by gender, age and body mass index, and GHD patients. However, Christ and colleagues (1997) reported that GH treatment in GHD adults (0.036 U/kg.day for three months) stimulated erythropoiesis similarly to the study by Kotzmann and colleagues (1996), who showed that after 24 months of treatment with GH to GH deficient adults there was a marked effect on erythroid and myeloid progenitor precursor cells.

The increase in the native aggregation of erythrocytes in the rhGH group might affect blood viscosity both at high shear rate (primarily relevant for flow in arteries, arterioles, and capillaries) and low shear rate (relevant for flow in veins and under stasis) (Vaya et al., 2009), in low-flow conditions, and may contribute to circulatory disorders and, perhaps in the microcirculation, to the occlusions of micro vessels (Luquita et al., 2009). According to Marossy and colleagues (2009) the main functions of blood are the transport and delivery of

oxygen and nutrients, removal of carbon dioxide and waste products of metabolism provided by circulation of the blood. Therefore, based upon our results, we might expect the administration of rhGH to healthy young males may lead to an acute detrimental effect on the supply of nutrients and O_2 for the muscle tissue of athletes in high energy demand performance.

Insulin Growth Factor-I (IGF-I) in serum increased significantly 24 hours after rhGH administration ($p \leq 0.001$) and remained unaltered in the placebo group, reflecting a very similar profile of the aggregation. This suggests that IGF-I has a role in the native aggregation of the RBC. The growth hormone–insulin growth factor-I axis is a regulator of blood rheology and in physiological conditions such as regular training in athletes it has the potential to alter blood rheology factors (Peyreigne et al., 1998).

4.2 Immune function and regulation

In the white blood cells (WBC) the ratio of $CD4^+/CD8^+$ T lymphocytes remained unaltered from baseline to 29 days after rhGH administration. Ramel and colleagues (2003) showed $CD4^+/CD8^+$ ratio to be decreased during submaximal resistance exercise at 75% of their 1 RM (repetition maximum) and increased during recovery. The resistance-trained participants tended to have lower T-helper cell counts before, during and immediately after exercise and a lower $CD4^+/CD8^+$ ratio during recovery than the non-resistance-trained participants. Some authors consider the lower $CD4^+/CD8^+$ ratio to contribute to the immune susceptibility of athletes after strenuous exercise, commonly performed by athletes while training or competing.

The T helper lymphocyte $CD4^+$ was also evaluated in our study as per activation (cytokine production), more specifically the production of cytokines such as, Interleukins 2, 4, 6 and 10, Interferon-γ and Tumour necrosis factor-α. With the exception of IL10, there was no

significant difference between or within groups from baseline to day 29. As shown in Study Two the late response of IL10 indicates a possible shift from Th1 to Th2 demonstrating an adaptive process by the immune system in an attempt to down regulate atypical inflammatory Th1 immune responses. Importantly, IL10 is well known for having essential immunoregulatory functions on the activity of immune cells with potent anti-inflammatory properties and suppresses the expression of inflammatory cytokines such as TNF-α, IL6 and IL1 from activated pro-inflammatory macrophages (Donnelly et al., 1999).

As previously reported in athletes, ultra distance (245 km) runs considerably increase plasma IL6 levels by 8000-fold (7781.0 ± 8317.3 pg/ml) immediately after (within 15 minutes) the end of the race, returning to baseline level only 48 hrs after the race (0.7 ± 0.5 pg/ml) (Margeli et al., 2005). Therefore GH might aid to balance the pro-inflammatory/anti-inflammatory system.

Growth hormone therapy over a period of 12 weeks was previously shown (Adamopoulos, 2003) in a randomized crossover comparison to mediate in the various stages of inflammatory processes in patients with idiopathic dilated cardiomyopathy. It was shown to significantly reduce ($p \leq 0.04$) pro-inflammatory cytokines such as IL6, as well as significantly increasing the ratio IL10/IL6. Similar results were shown in our study since IL10 significantly increased and IL6 remained the same in healthy young males. It might be seen as an advantage for athletes to decrease the pro-inflammatory responses to exercise due to rhGH administration.

In study two, there was no significant effect of rhGH on NK cell count, phenotype or activity. In contrast, Crist and colleagues (1987) found a positive relationship between IGF-I levels and the cytotoxic activity of NK cells and an increase in NK activity after exogenous GH

treatment in women with an impairment in GH secretion. In this case, the contradictory results are explained by the difference between impairment or normality of the GH secretion.

A later study confirmed that both total NK cell count and particularly $CD16^+$ NK cell subtype were reduced in GHD patients compared to controls (Sneppen et al., 2002). However, the studies that show significant results on NK cells after GH administration were performed over a period of at least ten weeks, suggesting the time course of GH replacement may be a key factor. Furthermore, improved lymphocyte functions such as NK cell activity was presented in GHD populations. This has not been confirmed in our study with young healthy non-GHD subjects. Perhaps the effects of rhGH on NK cells are regulatory and not pertinent in healthy population. A previous study with non-syndromic short children revealed GH receptor expression on immune cells (T, B and natural killer cells expressing $CD2^+$) was inversely related to the linear growth expression and the body mass index of subjects (Valerio et al., 1997).

4.3 Gene expression outcome

The late response of IL10 cytokine production (day 29) might be related to the concurrent IL10R mRNA profile expression. On day 29, IL10R expression presented the highest measure of a 4.57 fold increase in up-regulation in the rhGH group compared to the placebo group from baseline, as reported in Study Three. Although mRNA expression is not a measure of protein expression, in our results it may be indicative of it. The IL10R mediates IL10 activity, which acts mainly as anti-inflammatory or by managing inflammatory response, which is frequently seen in athletes during training or competition seasons.

The granzime A (GZMA) and perforin 1 (PFR1) presented a similar pattern of up-regulation associated to NFκB up-regulation, once more confirming a profile of increased anti-inflammatory responses in the rhGH group compared to placebo. A previous study (Connoll

et al., 2004) evaluated the effects of exercise (30 min of constant work rate cycle ergometry at 80% peak O_2 uptake) on the gene expression, measured via microarray. The GZMA gene expression reported a 2.0 fold increase from baseline to immediately after the exercise bout. However, from the end of exercise to 60 minutes post recovery a down-regulation to -2.97 fold change was observed. After 60 minutes into recovery, samples were down-regulated compared to the samples before exercise. This is considered a moment of risk for the athlete because of the low immune cell numbers and activity, referred to as the "open window theory" (Nieman, 2000).

According to our results, rhGH increases anti-inflammatory activation through GZMA, PRF1 and NFkB mRNA up-regulation in healthy young males. This suggests an advantage to athletes since being engaged in intensive periods of endurance training appears to make them more susceptible to minor infections (Gleeson, 2007).

A recent study showed that acute exercise activates the NFkB signalling pathway in rat skeletal muscle which leads to increased expression of enzymes involved in antioxidant defence (inducible nitric oxide synthase, endothelial nitric oxide synthase and mitochondrial Mn superoxide dismutase) (Ji et al. 2004). This is suggested as the underlying mechanism for adaptation to training (Gomes-Cabrera, 2006).

As shown by our results the possible effect of rhGH administration on genes related to immune function and regulation such as IL6 can be inhibited by NFκB, GZMA, PRF1, IL10R gene expression up-regulation. Even if the induction of mRNA is not always followed by an increase in the related protein expression or its activity, our results suggest that rhGH administration can possibly benefit athletes by up-regulating PRF1 mRNA as well as GZMA, IL10R, IL6R expression.

Miles and colleagues (2002) evaluated the perforin gene expression using quantitative RT–PCR from moderately trained runners (males from 18 to 40 years old) and compared it to controls before exercise, immediately after (0h), 1.5, 5, and 24 h following a 60 minutes run at 80% of VO_2 peak. The exercise bout decreased the *PFR1* gene expression in the runners group from before to after exercise and this decrease was maintained over 24 h.

4.4 Limitations of the study

This study respected the initial healthy status of its sample limiting the time of rhGH administration to a short-term of 7 days, where although no side effects were reported it is not a comparable dose to the ones suggested by the literature to be a explicitly lower than the doses used by athletes. Nevertheless, the dose applied in our study revealed consistent changes in the aspects evaluated.

It is noteworthy that the dose of rhGH administered was not based on the individual body weight or composition of subjects, being the same (1mg/day) for all the subjects, which could lead to different rhGH absorption due to different body composition.

In summary, this study encompassing with thirty healthy young males demonstrated some of the effects of rhGH administration to a healthy population such as the acute change in the blood rheology through the increased RBC aggregation, as well as the late activation of Th lymphocytes in the release of anti-inflammatory interleukins and at the molecular level reporting the increased mRNA expression of selected genes.

Chapter 5

Conclusions, Proposed Studies and Practical implications

5.1 Conclusion

According to the World Anti Doping Code (WADA, 2009), there are three main reasons, for including a substance in the list of prohibited substances: medical or other scientific evidence, pharmacological effect or experience that the substance or method, alone or in combination with other substances has the potential to enhance or enhances sport performance, the substance represents an actual or potential health risk to the athlete and that the use of the substance violates the spirit of sport described in the Code. At least two of these reasons were shown in our study.

As shown in this thesis, the short-term administration of rhGH lead to a negative effect on the blood circulation of a healthy population based on an increase in the native aggregation of erythrocytes defaulting the optimum flow of O_2. Based on the fact that GH is considered a major issue in the doping control among professional and amateur athletes, it is of crucial importance to report its effects on the blood rheology of healthy subjects. The observed increase in native aggregation is considered a determinant of the blood flow at low flow shear rates in the microcirculation possibly interfering with the regulation of O_2 supply to the tissues.

Furthermore, it is conclusive that short-term administration of rhGH can increase the expression of genes with anti-inflammatory action in PBMC of healthy young males suggesting a link between rhGH and molecular regulation of immune function in healthy population. It is portentous that rhGH may act on $CD4^+$T lymphocytes by increasing IL10 production and may then be a key component in T cell activation as the values of IL10 in athletes was previously reported to be lower than the healthy subjects' values (Dorofeyeva and Dorofeyev, 2004).

Additionally, immune function and cytokine production, as assessed by $CD4^+$ Th1 and Th2 cytokines, showed a chronic effect of rhGH to specific anti-inflammatory cytokine release, a possible advantage for the adjustment of the athletes' immunological status and consequently sports performance.

The results of this study demonstrated at the molecular level the effects of the short-term administration of rhGH in the up regulation of the anti inflammatory *IL10* gene pathway through the up regulation of *IL10R*. This suggests that the gene expression evaluation might be a sensitive way of investigating traces of the use of rhGH.

5.2 Proposed studies

Longer rhGH administration protocols may provide different results from the ones presented in this thesis as the acute effects on native aggregation noticed on Day 8. It suggests the possibility of extended increased aggregation and consequently decreased erythrocytes flow.

Perhaps longer duration of rhGH administration could generate a chronic GH negative feedback effect on its own secretion, predominantly through increased hypothalamic somatostatin secretion and may be important in the physiological control of pulsatile GH secretion.

The use of different doses of rhGH may affect in different ways the parameters evaluated in the three studies presented in this thesis. Blood viscosity was not evaluated in Study One but it could have been an important indicator of the detrimental effects of the increased native aggregation presented in this study.

Future studies should examine the effects of rhGH in the top level athletes since it is well shown by the literature that athletes present a very particular immune function with high level of inflammation reported. Whether rhGH can be an advantage for the immune function of

this population, it is to be studied. Further research should be undertaken to assess the role of *IL10* expression following rhGH biological pathway.

The B lymphocyte numbers and percentage, expression of CD19, remained unaltered throughout the study in both groups and the B lymphocyte activity was not evaluated. This should be evaluated in future studies since preciously Yoshida (1992) showed they have a direct stimulating effect on B lymphocytes.

Further investigation should evaluate important genes connected to Th lymphocytes since it was shown in this thesis that Th cytokines production was effectively altered by rhGH administration. Important genes related to immune system such as Tyrosine kinase 2 encodes a member of the JAK protein families, which was shown to be the pathway of action of GH. Cytotoxic T-lymphocyte-associated protein 4 (CTLA4), is a member of the immunoglobulin superfamily and encodes a protein which transmits an inhibitory signal to T cells. CTLA4 inhibits T lymphocyte activation by reducing IL2 release and expression, and by arresting T cells at the G1 phase of the cell cycle which is the interval between mitosis and DNA replication being characterized by cell growth (Appleman, 2000). Chemokine (C-X-C motif) receptor 3, this gene is prominently expressed in *in vitro* cultured effector/memory T cells, and in T cells present in many types of inflamed tissues. It is suggested that this gene and its cytokines participate in the recruitment of inflammatory cells.

5.3 Practical implications

The demonstrated effects of short-term administration of rhGH in a healthy population at the molecular level leads to a large margin of posterior consequences in the immune cells activation. This must be brought to the attention of coaches, athletes and sports professionals. This study provided evidence of the influence of rhGH on the immune system and on the regulation of mRNA expression of selected genes of healthy young males. Referred results

can lead to several outcomes since the evaluated variables of this study have a central role in the cascades of reactions in the immune function of the subjects and can be possibly applied to athletes.

Additionally, it should be noted that the acute effects reported at the RBC aggregation could negatively interfere with the athletes' performance in high O_2 demand situations.

In conclusion, the results discussed in this thesis show that there are beneficial effects of short-term administration of rhGH to healthy young males as it generates an anti-inflammatory response in the function of lymphocytes and up-regulation in the mRNA expression of selected genes related to immune system. However, the acute response of red blood cell aggregation raises a concern and is a potential threat to athletes' well being.

Appendices

1. **Poster Advertisement**
2. **Selection of subjects/volunteers**
3. **Subject Inclusion/Exclusion Questionnaire 2008**
4. **Pre-exercise screening system Sports Medicine Australia (SMA) 2005**
5. **Explanatory Statement**
6. **Participant Informed Consent**

1 - Growth Hormone Study

Strength Trained Subjects Required

This research is investigating the effects of Growth Hormone on muscular strength and anti-doping markers.

To be eligible subjects need to be:

- Healthy males between 18-35yrs
- Been resistance training at least 2-3 times per week for the previous 12 months
- Familiar with the bench press exercise.

For more information contact:

Sandra Ramos 0413 038 685 or

Dr Shane Rogerson 0415 498 099

2 - SELECTION OF SUBJECTS/VOLUNTEERS

CRITERIA TO BE USED

Subject Inclusion criteria:

1. Males aged 18-35 years

2. Have not used any doping substance or performance enhancing supplements in the previous 6 months. This excludes protein powder.

3. Body weight between 70-110 kg

4. A minimum of 1 year weight training experience

   Have been training at least 3 times a week for the previous 12 months.

   Experience with performing Bench Press exercise.

5. Australian citizen.

6. Participants will have a urine test to determine use of banned substances ie a drug test (IOC/WADA). If found positive they will be removed from the study.

Subject Exclusion Criteria:

1. Regular user of therapeutic and/or recreational drugs.

2. Contraindications or risk to exercise (recent injuries/risk factors for maximal strength testing etc).

3 - Subject Inclusion/Exclusion Questionnaire 2008

If you are unsure about any of the following questions please seek clarification before responding.

SUBJECTS NAME (please print)_____

DOB: _____

Current Bodyweight _____

1. In the last 12 months how many times per week do you resistance train?_____

2. Are you familiar with the Bench Press exercise? YES NO

3. Are you currently suffering from any chronic (long-term) injury that you feel may affect your ability to lift maximum loads during a bench press exercise or may affect your ability to maintain your training during the research period? Give details if relevant:

_____

_____

4. Are you prepared to maintain your current training throughout the research period (eg no changes to volume or intensity). YES NO

5. Do you smoke? YES NO

6. Have you used any performance enhancing drugs in the previous 12 months?

   YES NO

---

7. Do you use recreational drugs? YES NO

---

8. Are you currently taking any prescription medication? YES NO

---

9. Do you use any nutritional supplements? YES NO

---

10. Are you an Australian citizen YES NO

I have provided a truthful response to all of these questions (signed): Date: _____

Researcher_____ Date _____

Australian Government
Department of Health and Ageing

Sports Medicine Australia (SMA) pre-exercise screening system 2005

Introduction

Physical activity levels in the general community are low and decreasing (AIHW, 2004). The typical physical working capacity or ability to undertake prolonged moderate or vigorous exercise is poor. This is because in the absence of specific, dedicated exercise time, the majority of people in developed countries such as Australia are becoming increasingly sedentary at work and at home, have low energy expenditure in leisuretime pursuits and have low participation rates in active transport.

It is not uncommon for some people to go for many months or even years without undertaking any planned or structured physical activity. When these people decide to alter lifestyle patterns, join a gym or begin regular physical activity they are often unsure about how to be active. Unfortunately, through inappropriate exercise prescription or knowledge of the principles of progressive overload, **many people do too much too soon**. The result may be extreme muscle soreness or joint problems, or in rare cases they may place themselves at higher risk for acute cardiovascular problems. **For many people this is a demotivator and is related to the high dropout rates typically found for these new programs**.

Who is it for?

The Sports Medicine Australia (SMA) pre-exercise screening system is a tool for exercise professionals to use when deciding if a person is at a high risk for these problems and is therefore recommended for medical clearance before embarking on an exercise program. Also, the screening system helps to identify those at low or moderate levels of risk during exercise and directs them to begin a tailored physical activity program without the need to seek medical clearance. This is the most common route for the majority of the population. Undertaking regular physical activity is important for the health of everyone.

The SMA screening system is part of the broader effort to encourage physical activity. It is designed to provide a level of guidance so that those who are beginning regular physical activity are directed in an appropriate way to increase their safety and help them enjoy the experience. The SMA pre-exercise screening system is a modification of the American College of Sports Medicine's (ACSM) guidelines for pre-exercise screening and testing (ACSM, 2000). The ACSM guidelines are recognised as an important benchmark for the following reasons (Olds and Norton, 1999):

- The ACSM is an internationally recognised leader in the areas of exercise science and sports medicine
- The ACSM has produced six editions of their guidelines for pre-exercise screening and testing over the past 30 years which have been based on several decades of scientific, clinical and epidemiological research
- Similarities between Australian and North American populations in areas such as physical activity patterns, and morbidity and mortality statistics in lifestyle diseases such as cardiovascular disease, diabetes and cancer, justify their adaptation for use in Australia.

SMA pre-exercise screening system

1) The first stage of the screening system is a filter to screen out those people who are at a high risk level for exercise-related complications due to underlying cardiovascular, cerebrovascular, respiratory or metabolic diseases. These are people with known disease or who have signs and/or symptoms of disease. Other serious or potentially serious medical conditions that may be exacerbated during exercise are also important at this stage (see notes at 'Stage 1 - Overview' flow chart). It is recommended that this relatively small group of 'high risk' clients seek medical clearance before beginning an exercise program or undertaking aerobic fitness testing.

The questionnaire shown below is the tool used to identify who is at high risk at this stage.

- If a person answers 'Yes' to any of these questions then they are considered to be in the 'high risk' group. There is, however, scope for the exercise specialist to use a level of professional judgement when interpreting these responses. For example, swelling or fluid accumulation about the ankles may be related to local joint problems or recent air travel rather than, for example, due to underlying cardiovascular pathology. For those with well controlled diabetes or stable cardiovascular conditions (coronary heart disease (CHD), cardiac failure, stroke and peripheral vascular disease (PVD)) there is generally no need to seek medical clearance before beginning a low - moderate physical activity program such as regular walking (NHF 2005). Also, 'other' medical conditions that may be mentioned are essentially endless so there requires a level of interpretation in deciding if the risk of adverse effects outweighs the known benefits of individually-tailored regular physical activity.

Those who are NOT at high risk can begin low or moderate level physical activity without the need for medical clearance (see details below for specific guidelines and rare exceptions). These people may also proceed to stage 2 of the screening system if there is a desire to exercise at vigorous intensity levels or if there is an intention to undergo exercise testing to maximal levels.

2) The second stage of the screening system is used to determine those people who are categorised as moderate or low risk for exercise-related complications due to underlying cardiovascular, cerebrovascular, respiratory or metabolic diseases (or other medical conditions referred to below).

Stage 2 identifies those at moderate risk who are either 'older' and/or who have 2 or more risk factors for heart disease. For these clients they are classified as moderate risk and can undertake physical activity up to moderate intensity levels (for example, walking for the majority of people), without medical clearance. Stage 2 procedures and measures can also be used as a valuable adjunct in the general health appraisal of clients and to monitor changes in risk factor status over time and with lifestyle changes.

Those who are 'younger' and who have less than 2 risk factors are considered low risk for exercise-related complications. They can also be tested to maximal levels without medical clearance or supervision.

The general process for administering the pre-exercise screening system is illustrated below. Specific flow charts [decision-making trees] are then presented for each of the two stages.

Acknowledgment

This screening system was produced by Professor Kevin Norton of the University of South Australia in consultation with Sports Medicine Australia members.

References

American College of Sports Medicine (ACSM) (2000). ACSM's guidelines for exercise testing and prescription (6th ed). New York, Lippincott Williams & Wilkins.

Australian Institute of Health and Welfare (AIHW) (2004). Heart, stroke and vascular diseases Australian facts 2004. Canberra, AIHW and National Heart Foundation of Australia.

National Heart Foundation (NHF) (2005). Physical activity recommendations for people with cardiovascular disease. Sydney, National Heart Foundation of Australia.

Olds, T. and Norton, K. (1999). Pre-exercise health screening guide. Champaign, Ill, Human Kinetics.

Sports Medicine Australia (SMA)
National Office
Suite 3 Dickson Square
(PO Box 237) Dickson ACT 2602

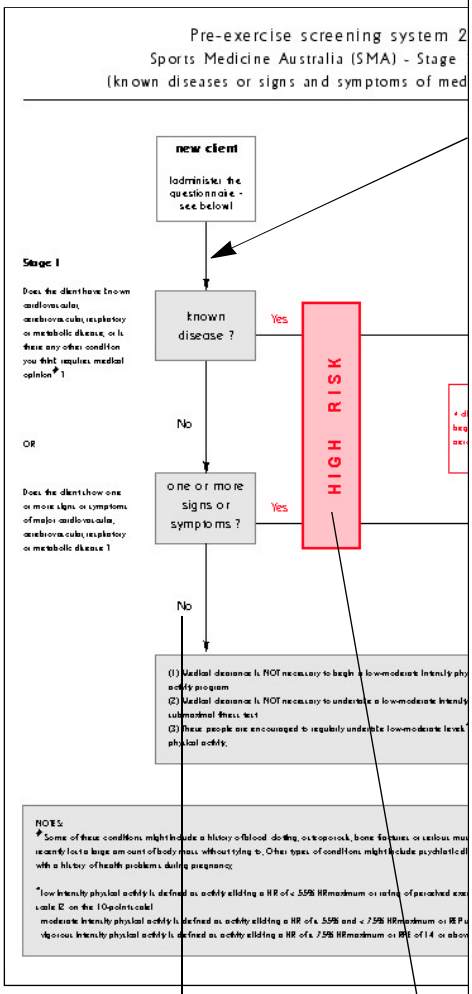
Tel 02 6230 4650
Fax 02 6230 5908
Email smanat@sma.org.au
website www.sma.org.au

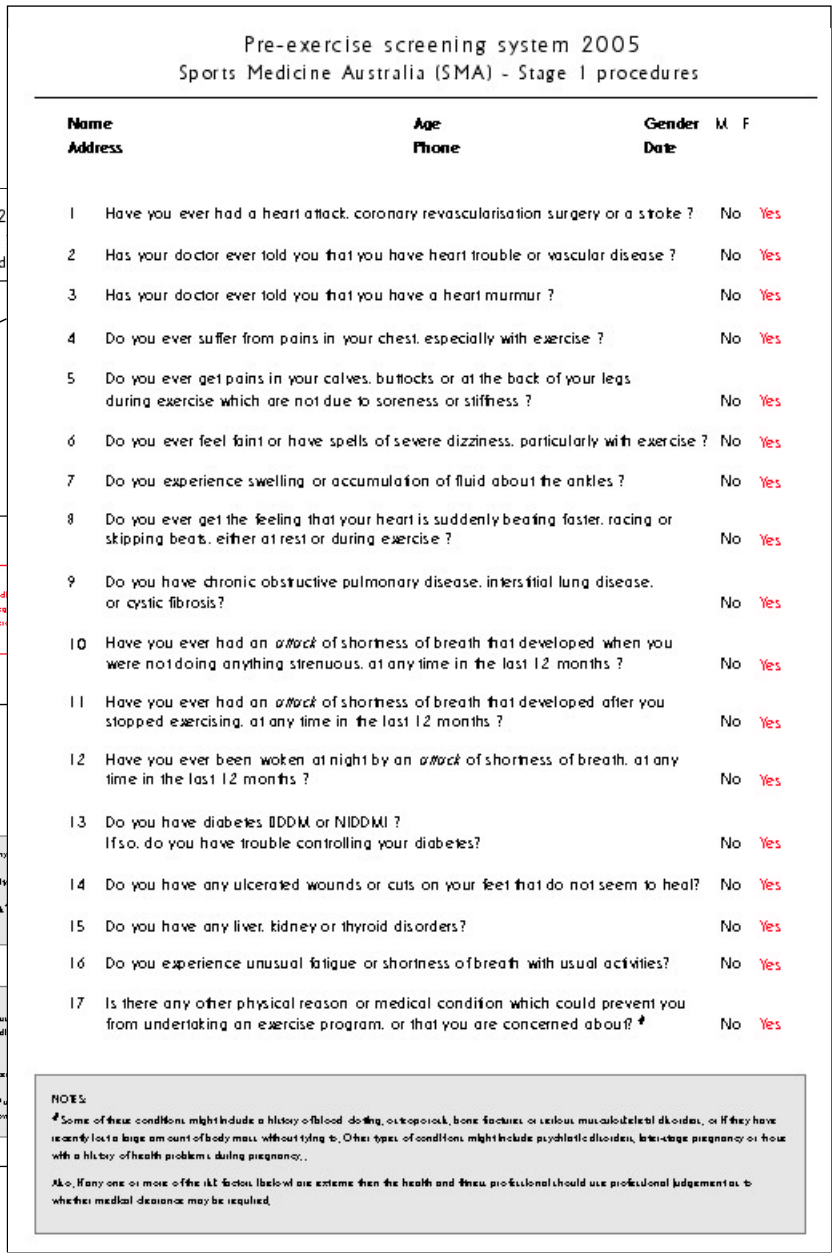
State Branches: see Website for details

STAGE 1 Administer the SMA pre-exercise screening questionnaire to new clients or those significantly upgrading their exercise activity to determine who is at a high risk

1A

administer QUESTIONNAIRE





Pre-exercise screening system 2005
Sports Medicine Australia (SMA) - Stage 1 procedures

Name **Age** **Gender** M F
Address **Phone** **Date**

1	Have you ever had a heart attack, coronary revascularisation surgery or a stroke ?	No	Yes
2	Has your doctor ever told you that you have heart trouble or vascular disease ?	No	Yes
3	Has your doctor ever told you that you have a heart murmur ?	No	Yes
4	Do you ever suffer from pains in your chest, especially with exercise ?	No	Yes
5	Do you ever get pains in your calves, buttocks or at the back of your legs during exercise which are not due to soreness or stiffness ?	No	Yes
6	Do you ever feel faint or have spells of severe dizziness, particularly with exercise ?	No	Yes
7	Do you experience swelling or accumulation of fluid about the ankles ?	No	Yes
8	Do you ever get the feeling that your heart is suddenly beating faster, racing or skipping beats, either at rest or during exercise ?	No	Yes
9	Do you have chronic obstructive pulmonary disease, interstitial lung disease, or cystic fibrosis?	No	Yes
10	Have you ever had an *attack* of shortness of breath that developed when you were not doing anything strenuous, at any time in the last 12 months ?	No	Yes
11	Have you ever had an *attack* of shortness of breath that developed after you stopped exercising, at any time in the last 12 months ?	No	Yes
12	Have you ever been woken at night by an *attack* of shortness of breath, at any time in the last 12 months ?	No	Yes
13	Do you have diabetes (IDDM or NIDDM) ? If so, do you have trouble controlling your diabetes?	No	Yes
14	Do you have any ulcerated wounds or cuts on your feet that do not seem to heal?	No	Yes
15	Do you have any liver, kidney or thyroid disorders?	No	Yes
16	Do you experience unusual fatigue or shortness of breath with usual activities?	No	Yes
17	Is there any other physical reason or medical condition which could prevent you from undertaking an exercise program, or that you are concerned about? #	No	Yes

NOTES:
Some of these conditions might include a history of blood clotting, osteoporosis, bone fractures or serious musculoskeletal disorders, or if they have recently lost a large amount of body mass without trying to, Other types of conditions might include psychiatric disorders, late-stage pregnancy or those with a history of health problems during pregnancy, .

Also, if any one or more of the risk factors (below) are extreme then the health and fitness professional should use professional judgement as to whether medical clearance may be required.

1B

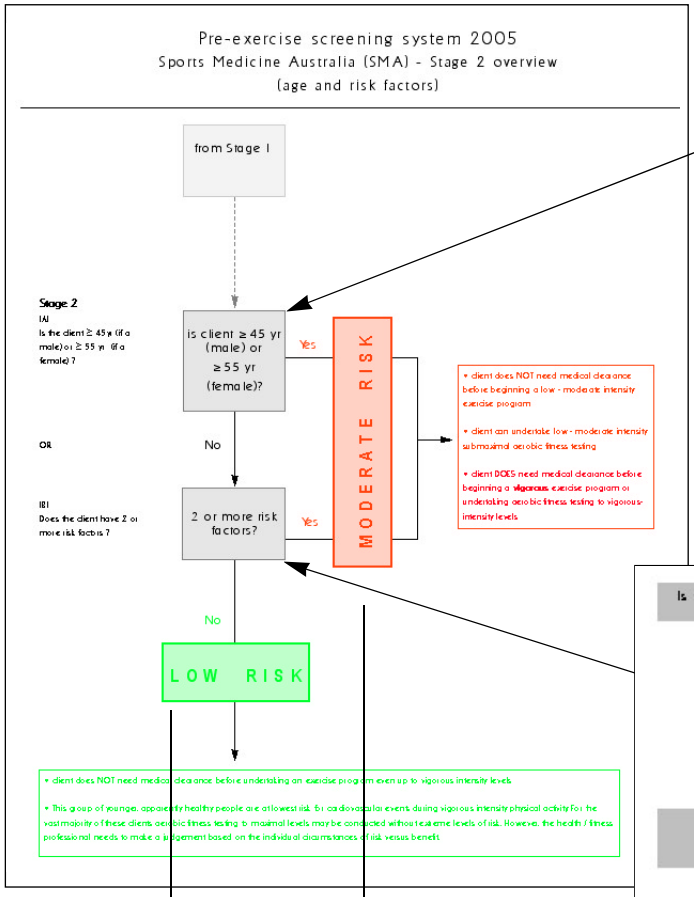
High risk clients should get medical clearance before beginning an exercise program or undergoing exercise testing

1C

Others can begin a low - moderate intensity physical activity program without the need for medical clearance*. For those people interested in commencing a vigorous intensity activity program or wanting to undergo exercise testing to maximal levels then proceed to stage 2

* see details below for specific guidelines and rare exceptions

STAGE 2 Use the (a) age categories and (b) cardiovascular risk factors to decide who is at a moderate or low level of risk

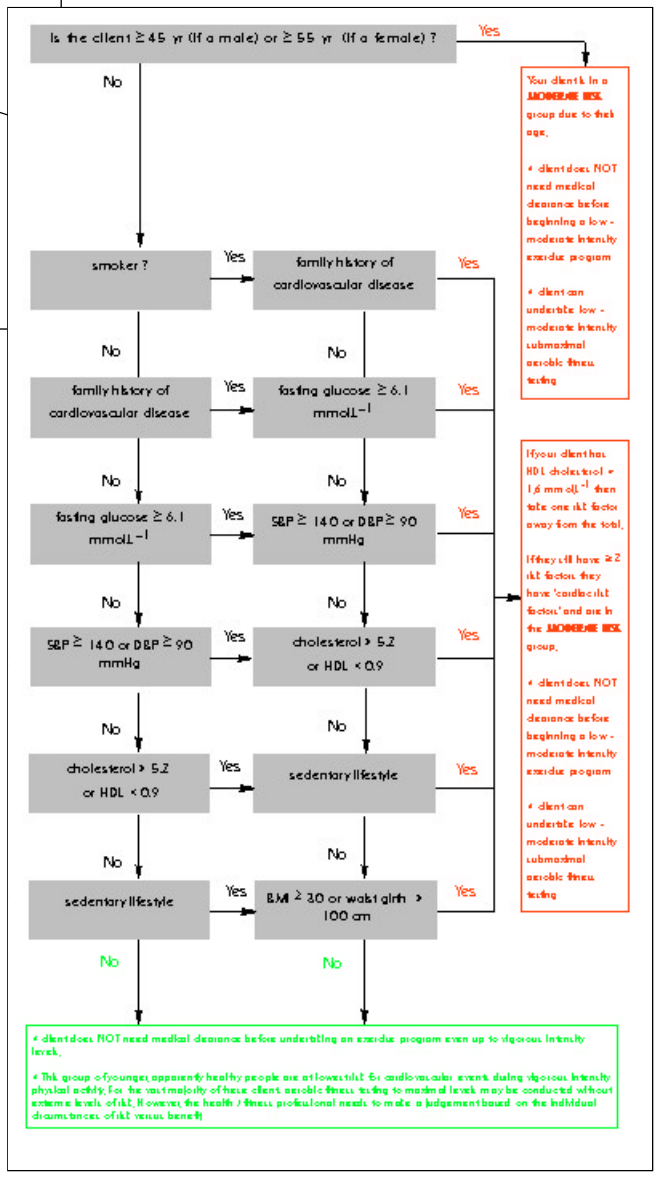


2A

is the client
≥ 45 yr (if male)
or
≥ 55 yr (if female)?

If YES then they are at a moderate risk for CHD

2B Check for RISK FACTORS



2C

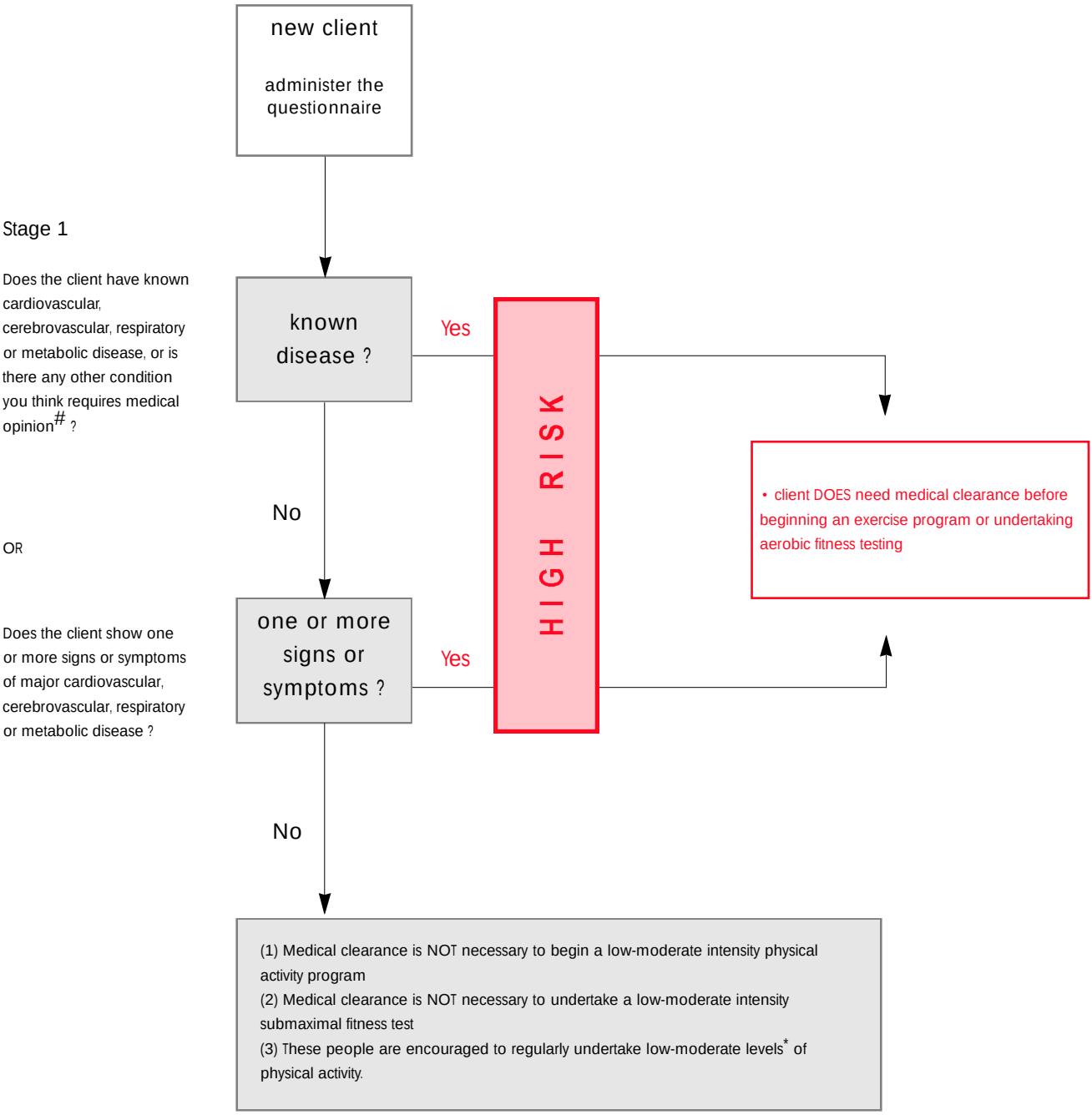
If the client is in the 'older' category OR if the client has 2 or more cardiac risk factors THEN they are at a moderate risk for CHD

Younger clients with less than 2 cardiac risk factors are at low risk for CHD

Pre-exercise screening system 2005

Sports Medicine Australia (SMA) - Stage 1 overview

(known diseases or signs and symptoms of medical conditions)



NOTES:

Some of these conditions might include a history of blood clotting, osteoporosis, bone fractures or serious musculoskeletal disorders, or if they have recently lost a large amount of body mass without trying to. Other types of conditions might include psychiatric disorders, later-stage pregnancy or those with a history of health problems during pregnancy. Those people taking medication(s) for medical conditions listed may also need medical clearance.

* low intensity physical activity is defined as activity eliciting a HR of < 55% HRmaximum or rating of perceived exertion [RPE] up to 11 on Borg's 20-point scale [2 on the 10-point scale]

moderate intensity physical activity is defined as activity eliciting a HR of ≥ 55% and < 75% HRmaximum or REP up to 13 [3 on the 10-point scale]

vigorous intensity physical activity is defined as activity eliciting a HR of ≥ 75% HRmaximum or RPE of 14 or above [5 on the 10-point scale]

Pre-exercise screening system 2005

Sports Medicine Australia (SMA) - Stage 1 questionnaire

Name | Age | Gender M F

Address | Phone | Date

1	Have you ever had a heart attack, coronary revascularisation surgery or a stroke ?	No	Yes
2	Has your doctor ever told you that you have heart trouble or vascular disease ?	No	Yes
3	Has your doctor ever told you that you have a heart murmur ?	No	Yes
4	Do you ever suffer from pains in your chest, especially with exercise ?	No	Yes
5	Do you ever get pains in your calves, buttocks or at the back of your legs during exercise which are not due to soreness or stiffness ?	No	Yes
6	Do you ever feel faint or have spells of severe dizziness, particularly with exercise ?	No	Yes
7	Do you experience swelling or accumulation of fluid about the ankles ?	No	Yes
8	Do you ever get the feeling that your heart is suddenly beating faster, racing or skipping beats, either at rest or during exercise ?	No	Yes
9	Do you have chronic obstructive pulmonary disease, interstitial lung disease, or cystic fibrosis?	No	Yes
10	Have you ever had an attack of shortness of breath that developed when you were not doing anything strenuous, at any time in the last 12 months ?	No	Yes
11	Have you ever had an attack of shortness of breath that developed after you stopped exercising, at any time in the last 12 months ?	No	Yes
12	Have you ever been woken at night by an attack of shortness of breath, at any time in the last 12 months ?	No	Yes
13	Do you have diabetes [IDDM or NIDDM] ? If so, do you have trouble controlling your diabetes?	No	Yes
14	Do you have any ulcerated wounds or cuts on your feet that do not seem to heal?	No	Yes
15	Do you have any liver, kidney or thyroid disorders?	No	Yes
16	Do you experience unusual fatigue or shortness of breath with usual activities?	No	Yes
17	Is there any other physical reason or medical condition, or are you taking any medication(s) which could prevent you from undertaking an exercise program, or that you are concerned about? #	No	Yes

NOTES:

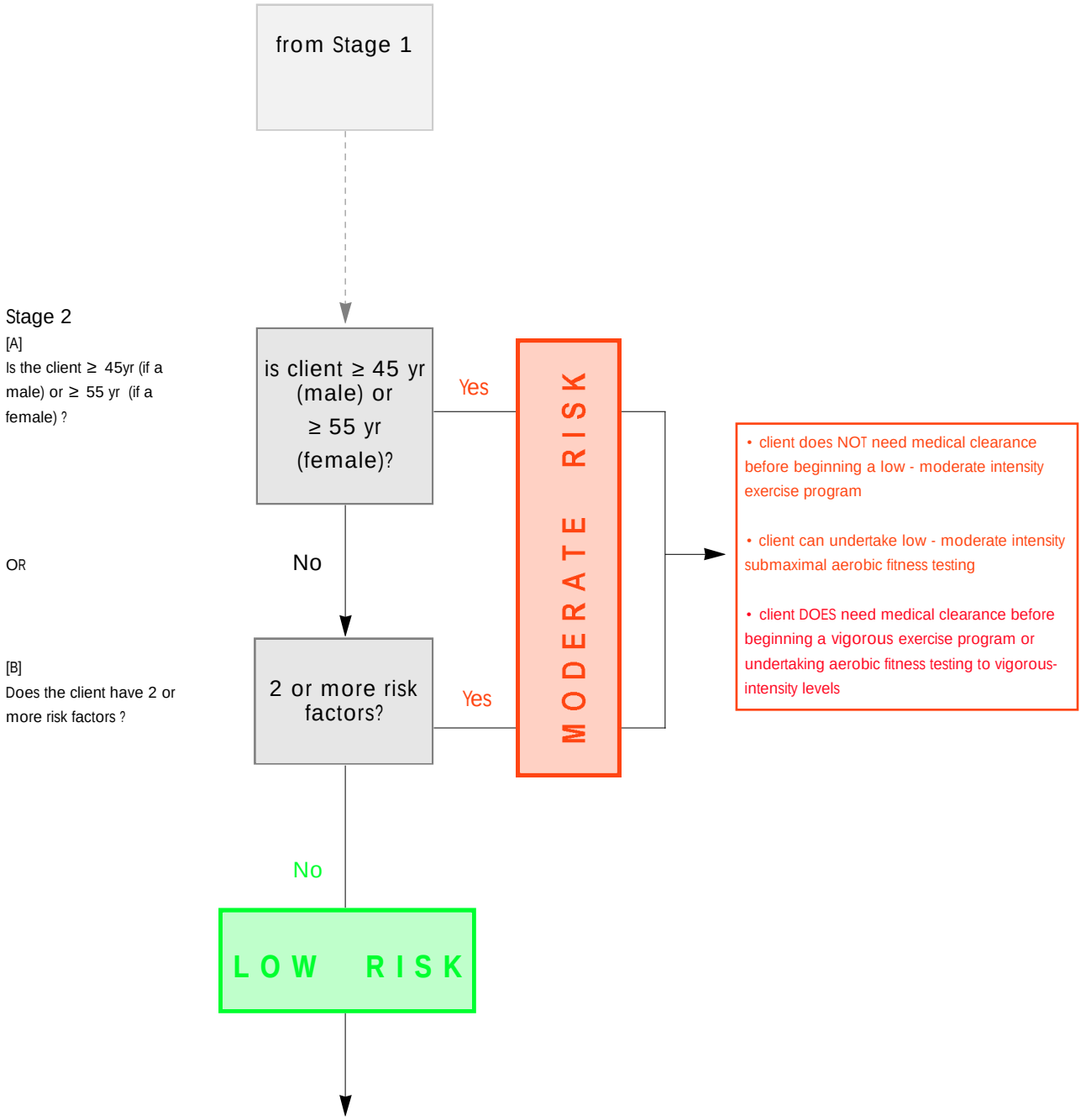
Some of these conditions might include a history of blood clotting, osteoporosis, bone fractures or serious musculoskeletal disorders, or if they have recently lost a large amount of body mass without trying to. Other types of conditions might include psychiatric disorders, later-stage pregnancy or those with a history of health problems during pregnancy. Those people taking medication(s) for medical conditions listed may also need medical clearance.

Also, if any one or more of the risk factors [below] are extreme then the health and fitness professional should use professional judgement as to whether medical clearance may be required.

Pre-exercise screening system 2005

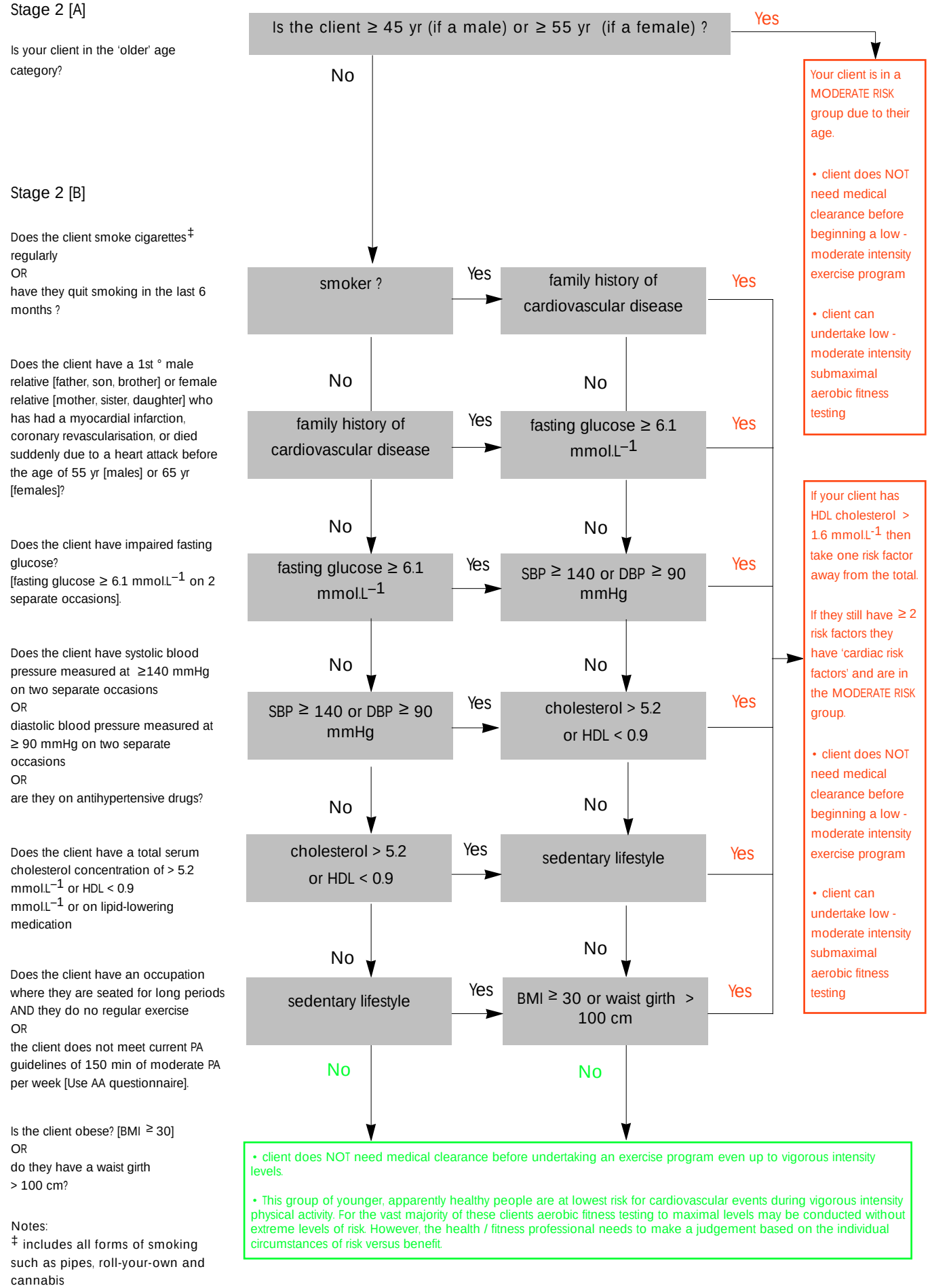
Sports Medicine Australia (SMA) - Stage 2 overview

(age and risk factors)



Pre-exercise screening system 2005

Sports Medicine Australia (SMA) - Stage 2 procedures



5 - Explanatory Statement

Project Title: **Transcriptional regulation of gene expression in human lymphocytes: Potential application to the detection of recombinant human Growth Hormone (rhGH): A one-year pilot project (Project Number: RO597)**

You are invited to participate in a study of the effects of Growth Hormone on the changes in gene expression within human white blood cells.

We hope to develop a test to detect the use of the banned substance, human recombinant Growth Hormone (rhGH) in athletes. This initial "pilot" project has been funded by the Anti-Doping Research Panel from the Commonwealth Department of Communication, Information Technology & the Arts. This project will investigate the effects of multiple doses (one dose per day for 7 days) of rhGH.

You were selected as a possible participant in this study because you are a resistance-trained male aged 18-35 years of age, NOT currently involved in competition.

If you decide to participate, you will be asked to visit the University to undertake familiarisation and initial screening where a medical history, height, and weight will be recorded. If you are admitted to the study you will be randomly allocated to one of two groups:

- Control (Placebo injection)
- Experimental Group – one injection of rhGH a day for 7 days.

At the commencement of the study, all subjects will have their 1RM (repetition maximum) for bench-press measured. Subsequently subjects will receive injections of either rhGH (Experimental Group) or Placebo (Control Group), according to the appropriate schedule. (as below)

- Control Group: (Injection of Placebo)
- Experimental Group: Daily injection of rhGH (1.0 mg) for seven (7) days, with collection of blood samples* on Days 0,1,8,15,22 & 29

All injections will be administered by a qualified and experienced medical practitioner, who will monitor subjects throughout the study.

*Note that blood sampling will involve venipuncture of a forearm vein and collection of 5-10mL of whole blood.

Subjects may experience some brief discomfort as the injection is administered. Possible adverse effects from administration of rhGH include oedema, muscle/skeleton/joint stiffness

or pain, or some sensory changes. Additional literature is available for subjects should they request any further information regarding the possible side-effects of rhGH. All subjects will be monitored by, and will have full access (24/7) to, the medical practitioner throughout the study. Participation of subjects in the study may be withdrawn should any adverse symptoms become apparent.

If you have any queries or would like to be informed of the aggregate research finding, please contact either:

Dr Bon Gray or;

Dr Shane Rogerson.

Faculty of Health Sciences & Medicine

Bond University – Gold Coast.

Queensland 4229.

Ph: 07 5595 4400 (SR) or 07 5595 4454 (BG)

Fx: 07 5595 4122.

Email: srogerson@bond.edu.au or bgray@bond.edu.au

Should you have any complaint concerning the manner in which this research is conducted, please do not hesitate to contact Bond University Research Ethics Committee, quoting the Project Number (above):

The Complaints Officer

Bond University Human Research Ethics Committee

Bond University Research and Consultancy Services

Level 2, Central Building

Bond University, QLD 4229.

Telephone (07) 5595 4194 Fax (07) 5595 1120

Email: buhrec@bond.edu.au

6- Participant Informed Consent

I agree to take part in the above Bond University research project. I have read the above Explanatory Statement and have been advised of any risks.

I agree to receive injections of either rhGH or Placebo and provide blood samples as outlined above.

_____ Please tick here to acknowledge that you understand and agree to the above procedures.

I understand that any information I provide is confidential, and that no information that could lead to the identification of any individual will be disclosed in any reports on the project, or to any other party.

I also understand that my participation is voluntary, that I can choose not to participate in part or all of the project, and that I can withdraw freely at any stage of the project.

Please tick the appropriate box

- ☐ The information I provide can be used by other researchers as long as my name and contact information is removed before it is given to them.

- ❑ The information I provide cannot be used by other researchers without asking me first.

- ❑ The information I provide cannot be used except for this project

Name: ..... (please print)

Signature: .....

Date: .....

Address:.....

.....

Phone Contact: ……..

Email Contact: …….

References

Adamopoulos S, Parissis, JT, Paraskevaidis I, Karatzas D, Livanis E, Georgiadis M, Karavolias M, Degiannis D, Kremastinos DT (2003) Effects of growth hormone on circulating cytokine network, and left ventricular contractile performance and geometry in patients with idiopathic dilated cardiomyopathy. *Eur Heart J* 24, 2186–2196.

Alberts B (2005). Leukocyte functions and percentage breakdown. Molecular Biology of the Cell. NCBI Bookshelf. Retrieved 2007-04-14.

Andiran N, Yordam N (2007). TNF-[alpha] levels in children with growth hormone deficiency and the effect of long-term growth hormone replacement therapy. Growth *Hormone & IGF Research*, 17(2), 149-153.

Appay V (2004) The physiological role of cytotoxic $CD4^+$ T-cells: the holy grail? *Clin Exp Immunol* 138:10–13.

Aquino Neto, F. R. d. (2001) O papel do atleta na sociedade e o controle de dopagem no esporte. *Revista Brasileira de Medicina do Esporte*, 7, 138-148.

Arndt V, Strassburg, CP, Manns, MP (2002) Genetic Association of Vitamin D receptor polymorphisms with primary biliary cirrhosis and autoimmune hepatitis. *Hepatology* 35, 126–131.

Arvat E, Di Vito L, Broglio F, Papotti M, Mucciolli G, Dieguez C, Casanueva Ff, Deghengi R, Camanni F, Ghigo E (2000) Preliminary evidence that Ghrelin, the natural GH secretagogue (GHS)-receptor ligand, strongly stimulates GH secretion in humans. *J Endocrinol Invest* 23: 493-495.

Asakawa K., Hedo, JA, McElduff, A., Rouiller, DG, Waters, MJ and Gorden, P. (1986) The human growth hormone receptor of cultured human lymphocytes. Structural characteristics and glycosylation properties. *Biochem J.* September 1; 238(2): 379–386.

Auernhammer, CJ, Strasburger, CJ (1995). Effects of growth hormone and insulin-like growth factor I on the immune system. *Eur J Endocrinol*, 133(6), 635-645.

Badolato R, Bond HM, Valerio G, Petrella A, Morrone G, Waters MJ, Venuta S, Tenore A. (1994) Differential expression of surface membrane growth hormone receptor on human peripheral blood lymphocytes detected by dual fluorochrome flow cytometry. *J Clin Endocrinol Metab.* 79(4):984-90.

Baechler EC, Batliwalla FM, Karypis G, Gaffney PM, Moser K, Ortmann WA, Espe KJ, Balasubramanian S, Hughes KM, Chan JP, Begovich A, Chang SY, Gregersen PK, Behrens TW (2004) Expression levels for many genes in human peripheral blood cells are highly sensitive to ex vivo incubation. *Genes Immun*. 5:347-353.

Baeza I, Alvarado C, Ariznavarreta C, Castillo C., Tresguerres JA, De la Fuente, M. (2008). Effect of growth hormone treatment on lymphocyte functions in old male rats. *Neuroimmunomodulation*, 15(4-6), 279-284.

Barbieri M, Ferrucci L, Ragno E, Corsi A, Bandinelli S, Bonafe M, Olivieri F, Giovagnetti S, Franceschi C, Guralnik JM, Paolisso G (2003) Chronic inflammation and the effect of IGF-I on muscle strength and power in older persons. *Am. J. Physiol. Endocrinol. Metab.* 284, E481–E487.

Barroso O, Mazzoni I, Rabin O. 2008 Hormone abuse in sports: the antidoping perspective. *Asian J Androl*; 10 (3): 391–402.

Bas A, (2004) Utility of the housekeeping genes 18S rRNA, beta-actin, GAPDH for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes. *Scan J Immunol.* Jun;59(6):566-73.

Basak S, Shih VFS, Hoffmann A (2008) Generation and Activation of Multiple Dimeric Transcription Factors within the NF-κB Signaling System *Mol Cell Biol*. May; 28(10): 3139–3150.

Baskurt OK, Yalcin O, Meiselman HJ. (2004) Hemorheology and vascular control mechanisms. *Clin Hemorheoland Micro* 30(3-4):169-78.

Baskurt OK, Meiselman HJ (2007) Hemodynamic effects of red blood cell aggregation. *Indian J. of Experim. Bio*. 45(1):25-31.

Berlato C, Cassatella MA, Kinjyo I, Gatto L, Yoshimura A, Bazzoni F (2002) Involvement of suppressor of cytokine signaling-3 as a mediator of the inhibitory effects of IL-10 on lipopolysaccharide-induced macrophage activation. *J Immunol.* Jun, 15;168(12):6404-11.

Bessis M, Mohandas N. (1975) A diffractometric method for the measurement of cellular deformability. *Blood Cells* 1:307-13.

Bouillanne O, Rainfray M, Tissandier O, Nasr A, Lahlou A, (1996) Growth hormone therapy in elderly people: an age-delaying drug? *Fundam Clin Pharmacol*. 10:416–430.

Boynard M, Lelievre JC (1987) Size determination of red blood cell aggregates induced by dextran using ultrasound backscattering phenomenon. Biorheology, 27, 39.

Brun, JF (2002) Hormone, metabolism and body composition as major determinants of blood rheology: Potential pathophysiologycal meaning. *Clin Hemorheol Micro* 26, 63-79.

Bryant J, Baxter L, Cave CB, Milne R. (2007) Recombinant growth hormone for idiopathic short stature in children and adolescents. *Cochrane Database of Systematic Reviews*, Issue 3 Art. No.: CD004440. DOI: 10.1002/14651858.CD004440.pub2.

Bull BS, Brailsford JD. (1976) Red cell membrane deformability: new data. Blood. Nov;48(5):663-7.

Butt FM, Vaghela VP, Chindia ML. (2007) Correlation of CD4 counts and CD4/CD8 ratio with HIV-infection associated oral manifestations. *East Afr Med J*;84: 383–8.

Caligiuri, MA (2008). Human natural killer cells. *Blood*, 112(3), 461-469.

Canty, TG, Wunderlich, JR (1970) Quantitative in vitro assay of cytotoxic cellular immunity J Natl Cancer Inst. 45:761.

Cappola AR, Xue QL, Ferrucci L, Guralnik JM, Volpato S, Fried LP (2003) Insulin-Like Growth Factor I and Interleukin-6 Contribute Synergistically to Disability and Mortality in Older Women *J Clin Endocrinol Metab*, 88(5):2019 –2025

Carter-Su C, King APJ, Smit LS, VanderKuur JA, Argetsinger LS, Campbell GS, Huo WJ (1997) Phosphorylation of highly purified growth hormone receptors by a growth hormone receptor-associated tyrosine kinase. *Anim. Sci.* 75 (Suppl. 2), 1 – 10.

Chelly J, Concordet JP, Kaplan JC, Kahn A (1989) Illegitimate transcription: transcription of any gene in any cell type. PNAS 86 2617–2621.

Chen HT, Schuler LA, Schultz RD (1998) Growth hormone receptor and regulation of gene expression in fetal lymphoid cells. *Mol Cell Endocrinol.* 137:21-29.

Chen, S., Barshtein G, Gavish B, Mahler Y, Yedgar S. (1994) Monitoring of red blood cell aggregability in a flow-chamber by computerized image analysis. *Clin. Hemorheol*. 14:497–508.

Cherwinski, HM, Schumacher, JH, Brown, KD, Mosmann, TR (1987). Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *J Exp Med*, 166(5), 1229-1244.

Chien S (1982) Rheology in the microcirculation in normal and low flow states. *Adv Shock Res* 8:71–80.

Clark R (1997) The somatogenic hormones and insulin-like growth factor-1: stimulators of lymphopoiesis and immune function. *Endocr Rev.* 18:157-179.

Clauss A (1957) Gerinnungsphysiologische Schnellmethode zur Bestimmung des Fibrinogens. *Acta Haematol*. 17:237–241.

Claustres M, Chatelain P, Sultan C (1987) Insulin-like growth factor I stimulates human erythroid colony formation *in vitro. J Clin Endocrinol Metab*65:78–82.

Cooper MD, Alder MN (2006) The evolution of adaptive immune systems, *Cell* 124, 815-822.

Crist, D. M., Peake, G. T., Mackinnon, L. T., Sibbitt, W. L., Jr., & Kraner, J. C. (1987). Exogenous growth hormone treatment alters body composition and increases natural killer cell activity in women with impaired endogenous growth hormone secretion. Metabolism, 36(12), 1115-1117.

Connolly, PH, Caiozzo, VJ Zaldivar F, Nemet D, Larson J, Hung S, Heck JD, Hatfield GW, Cooper DM (2004) Effects of exercise on gene expression in human peripheral blood mononuclear cells. *J Appl Physiol* 97: 1461–1469

Christ ER, Cummings MH, Westwood NB, Sawyer BM, Pearson TC, Sonksen, PH, Russell-Jones DL (1997) The Importance of Growth Hormone in the Regulation of Erythropoiesis, Red Cell Mass, and Plasma Volume in Adults with Growth Hormone Deficiency *E. R. J. Clin. Endocrinol and Metab* Vol 82 No 9.

D'Acquisto F, May MJ, Ghosh S. (2002) Inhibition of nuclear factor kappa B (NFB): an emerging theme in anti-inflammatory therapies. *Mol Interv*, 2:22-35.

Dardenne M, Mello-Coelho V, Gagnerault MC, Postel Vinay MC (1998) Growth hormone receptors and immunocompetent cells. *Ann. NY Acad. Sci.* 840, 510.

Daughaday WH, Rotwein P. (1989) Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum and tissue concentrations. *Endocr Rev*.; 10: 68–91.

Demiroglu H, Yalcin, Buyukasik Y, Ozcebe OI, Dundar S. (1998) Increased erythrocyte aggregation as an indicator for an aggressive clinical course in Behçet's disease: a prospective study *Ann Rheum Dis.* November; 57(11): 694–696.

Dendorfer U (1996) Molecular biology of cytokines. *Artif Organs* 20:437–444.

de Vos A, Ultsch M, Kossiakoff AA (1992) Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. *Science* 255:307–312.

Dimitrov S, Lange T, Fehm HL, Born J. (2004) A regulatory role of prolactin, growth hormone, and corticosteroids for human T-cell production of cytokines. *Brain Behav Immun* 18(4):368-74.

Dobbe JGG, Streekstra GJ, Hardeman MR, Ince C, Grimbergen CA (2002) Measurement of the distribution of red blood cell deformability using an automated rheoscope. *Cytometry* (50), 313-325.

Donnelly RP, Dickensheets H, Finbloom DS. (1999) The interleukin-10 signal transduction pathway and regulation of gene expression in mononuclear phagocytes. *J Interferon Cytokine Res.*;19:563-73.

Dorofeyeva EE, Dorofeyev AE (2004) Biochemical and heart adaptations to physical training and supplementation with amino acids. *J Strength Cond Res*. Nov;18(4):738-40.

El-Sayed MS, Ali N, El-Sayed Ali Z. (2005) Haemorheology in exercise and training. *Sports Med.* 35(8):649-70.

Engstrom KG, Ohlsson L, Oscarsson J (1990) Effect of hypophysectomy and growth hormone substitution on red blood cell morphology and filterability in rats. *J Lab Clin Med* 116:196–205.

Espy, MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA Yao JDC Wengenack, N. L, Rosenblatt JE, Cockerill FR III, Smith TF (2006) Real-Time PCR in Clinical Microbiology: Applications for Routine Laboratory Testing. *Clinical Microbiology Reviews*. 19(1):165-256.

Evans DM, Zhu G, Duffy DL, Frazer IH, Montgomery GW, Martin NG (2004) A major quantitative trait locus for CD4–CD8 ratio is located on chromosome 11. *Genes and Immunity* 5, 548–552

Fainaru-Wada M, Williams L (2006) Game of Shadows: Barry Bonds, BALCO, and the Steroids Scandal that Rocked Professional Sports. Publisher: Gotham Books; 1st edition.

Professional Sports (Gotham 2006)

Farag, SS, Fehniger TA, Ruggeri L, Velardi A, Caligiuri MA (2002) Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. *Blood* 100: 1935-1947.

Fazio, S., Sabatini D, Capaldo B, Vigorito C, Giordano A, Guida R, Pardo F, Biondi B, Saccà L (1996). A Preliminary Study of Growth Hormone in the Treatment of Dilated Cardiomyopathy. *New Engl J Med* 334(13): 809-814.

Finbloom DS, Winestock KD. (1995) IL-10 induces the tyrosine phosphorylation of Tyk2 and Jak1 and the differential assembly of STAT1 and STAT3 complexes in human T cells and monocytes. *J Immunol*;155:1079-90.

Fruhman GJ, Gerstner R, Gordon AS (1954) Effects of Growth Hormone upon Erythropoiesis in the Hypophysectomized Rat. *Exp Biol Med* 85, 93-96.

Fu, Y. K., Arkins, S., Fuh, G., Cunningham, B. C., Wells, J. A., Fong, S., et al., (1992). Growth hormone augments superoxide anion secretion of human neutrophils by binding to the prolactin receptor. *J Clin Invest* 89(2), 451-457.

Gagnerault MC, Touraine P, Savino W, Kelly PA, Dardenne M (1993) Expression of prolactin receptors in murine lymphoid cells in normal and autoimmune situations. *The Journal of Immunology*, vol. 150 (12), 5673-5681.

Gala RR, Shevach EM. (1993) Influence of prolactin and growth hormone on the activation of dwarf mouse lymphocytes in vivo. *Proc Soc Exp Biol Med* 204:224–230.

Gaytan, F., Romero, J. L., Bellido, C., Morales, C., Reymundo, C., & Aguilar, E. (1994). Effects of growth hormone and prolactin on testicular macrophages in long-term hypophysectomized rats. *J Reprod Immunol*, 27(1), 73-84.

Geffner ME, Bersch N, Lippe BM, et al., (1990) Growth hormone mediates the growth of T-lymphoblast cell lines via locally generated insulin-like growth factor-I. *J Clin Endocrinol Metab*. Aug;71(2):464-9.

Gladkevich A, Nelemans SA, Kauffman HF, Korf J (2005) Microarray Profiling of lymphocytes in internal diseases with an altered immune response: potential and methodology. Mediators of Inflammation, 317-320.

Gleeson M. (2007) Immune function in sport and exercise. J Appl Physiol 103: 693–699.

Gnanapavan, S., Kola B, Bustin SA, Morris DG, McGee P, Fairclough P, Bhattacharya S, Carpenter R, Grossman AB, Korbonits M (2002) The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. J. Clin. Endocrinol. Metab. 87:2988

Golde DW, Bersch N, Li CH 1977 Growth hormone: species-specific stimulation of erythropoiesis in vitro. *Science* 196 1112–1113.

Gregersen PK. (2003) Teasing apart the complex genetics of human autoimmunity: lessons from rheumatoid arthritis. *Clin Immunol* 107: 1–9.

Guler HP, Zapf J, Schmid C, Froesch ER (1989) Insulin-like growth factors I and II in healthy man. Estimations of half-lives and production rates. *Acta Endocrinologica* 121 753–758.

Hardeman MR, Goedhart PT, Dobbe JGG, Lettinga KP (1994) Laser-assisted optical rotation cell analyzer (LORCA). I. A new instrument for measurement of various structural hemorheological parameters. *Clin Hemorheol* 14:605–619.

Hasnain M. Khandwala, Ian E. MCcutecheon, Allan Flyvbjerg and Keith E. Friend (2000) The Effects of Insulin-Like Growth Factors on Tumorigenesis and Neoplastic Growth *Endocr Rev*21(3): 215–244

Haffner, D, Schaefer F, Nissel R, Wühl E, Tönshoff B, Mehls O, (2000) Effect of Growth Hormone Treatment on the Adult Height of Children with Chronic Renal Failure. *New England Journal of Medicine* 343(13): 923-930.

Hanley MB, Napolitano LA, McCune JM (2005) Stimulation of Hematopoiesis by Growth Hormone. *Stem Cells*; 23:1170–1179.

Henschen A, Lottspeich,F (1977) Amino acid sequence of human fibrin. Preliminary note on the completion of the beta-chain sequence. *Hoppe Seylers.Z. Physiol Chem* 358, 1643-1646.

Herrington J, Smit LS, Schwartz J, Carter-Su C (2000) The role of STAT proteins in growth hormone signalling. *Oncogene* 19, 2585-2597.

Higgins AJ (2004) Fat versus lean: the quest for beautiful buttocks. *The Veterinary Journal*, 167(3): 217-218.

Houbouyan LL, Delamaire M, Beauchet A, Gentil M, Cauchois G, Taccoen A, Yvert JP, Montredon N, Roudaut MF, Zhao S, Goguel A, Potron G, Boisseau M, Stoltz JF (1998) Multicenter study of an erythro-aggregameter: quality control and standardization. *Clin Hemorheol Microcirc* 17, 299.

Holt RIG and Sonksen PH (2008) Growth hormone, IGF-I and insulin and their abuse in sport *Br. J.of Pharmacol.*154, 542–556.

Hussein MR, Fathi NA, El-Din AM, Hassan HI, Abdullah F, Al-Hakeem E, et al., (2008) Alterations of the CD4(+), CD8 (+) T cell subsets, interleukins-1beta, IL-10, IL- 17, tumor necrosis factor-alpha and soluble intercellular adhesion molecule-1 in rheumatoid arthritis and osteoarthritis: preliminary observations. *Pathol Oncol Res*;14:321–8.

Jones JI, Clemmons DR (1995) Insulin-like growth factrs and theirs binding proteins: biological actions. *Endocr. Rev*. 16: 3.

Ji LL, Gomez-Cabrera MC, Steinhafel N, Vina J (2004) Acute exercise activates nuclear factor (NF) kB signaling pathway in rat skeletal muscle. *Faseb J* 18, 1500 –1506.

Johannsson G, Rosen T, Lindstedt G, Bosaeus I, Bengtsson BA (1996) Effect of 2 years of growth hormone treatment on body composition and cardiovascular risk factors in adults with growth hormone replacement therapy. *Endocrinol Metab* 4 (Suppl A):3-12.

Kabe Y, Ando K, Hirao S, Yoshida M, Handa H. (2005) Redox regulation of NFkappaB activation: distinct redox regulation between the cytoplasm and the nucleus. *Antioxid Redox Signal*, 7:395-403.

Kesmarky G, Toth K, Habon L, Vajda G, Juricskay I. (1998) Hemorheological parameters in coronary artery disease. *Clin Hemorheol Microcirc.* 18(4):245-51.

Kamm, RD. (2002) Cellular fluid mechanics. Ann Rev Fluid Mech, 34:211-32.

Kelley KW, Weigentc DA, Kooijmand R (2007) Protein Hormones and Immunity Brain Behav Immun. 21(4): 384–392

Khandwala HM, Mccutcheon IE, Flyvbjerg A, Friend KE The Effects of Insulin-Like Growth Factors on Tumorigenesis and Neoplastic. *Growth Endocrine Reviews* 21(3): 215–244.

Kiess W, Butenandt O (1985) Specific growth hormone receptors on human peripheral mononuclear cells: reexpression, identification and characterization. *J Clin Endocrinol Metab* 60:740 –746.

Kimata H, Yoshida A. (1994) Differential effect of growth hormone and insulin-like growth factor-I, insulin-like growth factor-II, and insulin on Ig production and growth in human plasma cells. *Blood* Mar 15;83(6):1569-74.

Kleinert H, Pautz A, Linker K, Schwarz PM (2004) Regulation of the expression of inducible nitric oxide synthase. *Eur J Pharmacol*;500:255–66.

Kramer HF, Goodyear LJ (2007) Exercise, MAPK, and NF-κB signalling in skeletal muscle. *J Appl Physiol.* 103:388–395.

Kojima M, Hosoda H, Date Y (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 402: 656-660.

Kooijman R, Willems M, De Haas CJ, Rijkers GT, Schuurmans AL, Van Buul- Offers SC, et al., (1992). Expression of type I insulin-like growth factor receptors on human peripheral blood mononuclear cells. *Endocrinology,* 131(5), 2244-2250.

Kotzmann H, Riedl M, Clodi M, Barnas U, Kaider A, Höcker P, Luger A. (1996) The influence of growth hormone substitution therapy on erythroid and myeloid progenitor cells and on peripheral blood cells in adult patients with growth hormone deficiency. *Eur J Clin Invest* Dec 26 (12):1175-81.

Lai CF, Ripperger J, Morella KK, Jurlander J, Hawley TS, Carson WE, Kordula T, Caligiuri MA, Hawley RG, Fey GH, Baumann H (1996) Receptors for interleukin (IL)-10 and IL-6-type cytokines use similar signaling mechanisms for inducing transcription through IL-6 response elements. *J. Biol. Chem.* Vol. 271, No. 24, Issue of June 14, pp. 13968 –13975.

Lappas M, Permezel M, Georgiou HM, Rice GE. (2002) Nuclear factor kappa B regulation of proinflammatory cytokines in human gestational tissues *in vitro*. *Biol Reprod*, 67:668-73.

Larsson, B., Svardsudd, K., Welin, L., Wilhelmsen, L., Bjorntorp, P. and Tibblin, G. (1984) Abdominal adipose tissue distribution, obesity, and risk of cardiovascular disease and death: 13 year follow up of participants in the study of men born in 1913. *British medical journal* (Clinical Research ed.) 288, 1401-1404.

Lecoeur H, Février M, Garcia S, Riviere H, Gougeon, ML (2001) A novel flow cytometric assay for quantitation and multiparametric characterization of cell-mediated cytotoxicity. *J Immunol Methods* 253(1-2): 177-187.

Lesniak MA, Gorden P, Roth J, Gavin Jr I (1974) Binding of 125I human growth hormone to specific receptors in human cultured lymphocytes. *J Biol Chem* 249:1661–1667.

Li, N., L. Zhou, Zhang B, Dong P, Lin W, Wang H, Xu R, Ding H (2008) Recombinant human growth hormone increases albumin and prolongs survival in patients with chronic

liver failure: A pilot open, randomized, and controlled clinical trial. *Digestive and Liver Disease* 40(7): 554-559.

Liu PK, Goudreau B, Hsu GS. (1989) Aphidicolin hypersensitive mutant of Chinese hamster V79 fibroblasts that underproduces DNA polymerase-alpha antigen. *Somat Cell Mol Genet*. 15 (4):331-44.

Liu Y, Wei SH, Ho AS, De Waal MR, Moore KW. (1994) Expression cloning and characterization of a human IL-10 receptor. *J Immunol*;152:1821.

Lockhart DJ, Winzeler EA (2000) Genomics, gene expression and DNA arrays. *Nature* Vol. 405, 827- 836.

Loesch K, Deng L, Cowan JW, Wang X, He K, Jiang J, Black RA, Frank SJ (2006) Janus Kinase 2 Influences Growth Hormone Receptor Metalloproteolysis. *Endocrinology* 147:2839-2849.

Lowe GDO, Drummond MM, Lorimer AR, Hutton I, Forbes CD, Prentice CRM, Barbenel JC (1980) Relation between extent of coronary artery disease and blood viscosity. *BMJ* 290:673–674.

Lowe GD, Rumley A, Mackie IJ. (2004) Plasma fibrinogen. *Ann Clin Biochem*;41:430–40.

Luquita A, Urli L, Svetaz MJ, Gennaro AM, Volpintesta R, Palatnik S, Rasia M. (2009) Erythrocyte aggregation in rheumatoid arthritis: Cell and plasma factor's role *Clin Hemorheol Micro* (41); 49–56.

Maas K, Chan S, Parker J, Slater A, Moore J, Olsen N, Aune TM (2002) Cutting Edge: Molecular Portrait of Human Autoimmune Disease *J Immunol*169: 5-9.

Maeda N, (1996) Erythrocyte Rheology in Microcirculation, *JPN J Physiol,* Vol. 46, 1-14.

Margeli A, Skenderi K, Tsironi M, Hantzi E, Matalas AL, Vrettou C, Kanavakis E, Chrousos G, Papassotiriou I (2005) Dramatic Elevations of Interleukin-6 and Acute-Phase Reactants in Athletes Participating in the Ultradistance Foot Race Spartathlon: Severe Systemic Inflammation and Lipid and Lipoprotein Changes in Protracted Exercise. *The Journal of Clinical Endocrinology & Metabolism*, vol. 90; 7, 3914-3918.

Marossy A, Svorc P, Kron I, Gresová S. (2009) Haemorheology and circulation. *Clin Hemorheol Microcirc*; 42(4): 239-58.

Marshall-Gradisnik SM, Weatherby RP, Deakin G, Coutts RA, Meir RA, Connellan PA, et al., (2008). Natural killer cell activity following six weeks of strength training in healthy young males with/without testosterone enanthate administration: *Journal of Exercise Science and Fitness*, vol. 6, no. 2, pp. 106-114.

Mathews LS, Enberg B, Norstedt G (1989) Regulation of rat growth hormone receptor gene expression. *Journal of Biological Chemistry* 264 9905–9910.

Merchav S, Tatarsky I, Hochberg Z. (1988) Enhancement of erythropoiesis *in vitro* by human growth hormone is mediated by insulin-like growth factor I. *Br J Haematol* Nov;70(3):267-71.

Melmed S. (2006) Medical progress: Acromegaly. *N Engl J Med.* 14; 355(24): 2558-73.

Merchav, S., Tatarsky, I., & Hochberg, Z. (1988). Enhancement of erythropoiesis in vitro by human growth hormone is mediated by insulin-like growth factor I. *Br J Haematol*, 70(3), 267-271.

Mercola KE, Cline MJ, Golde DW. (1981) Growth hormone stimulation of normal and leukemic human T-lymphocyte proliferation *in vitro*. *Blood.* Aug;58(2):337-40.

Miles MP, Mackinnon LT, Grove DS, Williams NI, Bush JA, Marx JO, Kraemer WJ, Mastro AM (2002) The relationship of natural killer cell counts, perforin mRNA and CD2

expression to post-exercise natural killer cell activity in humans. *Acta Physiologica Scandinavica* Volume 174, Issue 4, pages 317–325.

Miljic D, Miljic P, Doknic M, Pekic S, Djurovic M, Colovic M, Popovic V (2006) Changes in prothrombin and activated partial thromboplastin time during replacement therapy with human recombinant growth hormone in growth hormone deficient adults *Hormones*, 5(3):187-191.

Mohandas N, Clark MR, Jacobs MS, Groner W, Shohet SB. (1980) Ektacytometric analysis of factors regulating red cell deformability. *Blood Cells*, 6: 329–334.

Monnier JF, Aïssa Benhaddad A, Micallef JP, Mercier J and Brun JF (2000) Relationships between blood viscosity and insulin-like growth factor I status in athletes. *Clin Hemorheol Microcirc.* 22 277–286.

Moore KW, de Waal Malefyt R, Coffman, RL, O'Garra A (2001). Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol*, 19, 683-765.

Mosmann TR, Coffman RL. (1989) Heterogeneity of cytokine secretion patterns and functions of helper T cells. *Adv Immunol.*;46:111-47.

Mosmann, T, Sad, S. (1996) The expanding universe of T-cell subsets: Th1, Th2, and more. *Immunol Today* 17:138.

Mozaffari F, Hansson L, Kiaii S, Ju X, Rossmann ED, Rabbani H, et al., (2004). Signalling molecules and cytokine production in T cells of multiple myeloma-increased abnormalities with advancing stage. *Br J Haematol*, 124(3), 315-324.

Murphy WJ, Durum SK, Longo DL (1992) Role of neuroendocrine hormones in murine T cell development. Growth hormone exerts thymopoietic effects in vivo. *J Immunol* 149:3851–3857.

Murphy K, Travers P, Walport M (2007) Janeway's Immunobiology. New York: Garland Science.

Murphy K, Travers P, Walport M (2008) Janeway's Immunobiology. New York: Garland Science.

Musielak M (2009) Red cell-deformability measurement: review of techniques. *Clin Hemorheol Microcirc* 41 (1), 47-64.

Napolitano LA, Schmidt D, Gotway MB, Ameli N, Filbert EL, Ng MM, Clor JL, Epling L, Sinclair E, Baum PD, Li K, Killian ML, Bacchetti P, McCune JM (2008) Growth hormone enhances thymic function in HIV-1-infected adults. *J Clin Invest*, 118:1085-1098.

Neri S, Mariani E, Meneghetti A, Cattini l, Facchini A (2001) Calcein-Acetyoxymethyl Cytotoxicity Assay: Standardization of a Method Allowing Additional Analyses on Recovered Effector Cells and Supernatants *Clin. Diagn. Lab. Immunol.,* 8: 1131 - 1135.

Nieman, DC (2000) Special feature for the olympics: Effects of exercise on the immune system: Exercise effects on systemic immunity. *Immunol Cell Biol*, 78(5), 496 – 501.

Omoigui S (2007) The Interleukin-6 inflammation pathway from cholesterol to aging – Role of statins, bisphosphonates and plant polyphenols in aging and age-related diseases. *Immunity & Ageing*, 4:1

Orphanides, G, Reinberg D (2002) A Unified Theory of Gene Expression. *Cell* 108(4): 439-451.

Nussey SS, Whitehead SA. (2001) Endocrinology: an integrated approach. In London: BIOS Scientific Publishers Limited, 358pp.

Nyman T, Pekonen F. (1993) The expression of insulin-like growth factors and their binding proteins in normal human lymphocytes. *Acta Endocrinol* (Copenh)128(2):168-72.

Orphanides G, Reinberg, D (2002) A unified theory of gene expression. *Cell* 108: 439–451.

Pahwa S, Read JS, Yin W, Matthews Y, Shearer W, Diaz C, et al., (2008) CD4+/CD8+ T cell ratio for diagnosis of HIV-1 infection in infants: Women and Infants Transmission Study. P*ediatrics;*122:331–9.

Pan MH, Lin-Shiau SY, Lin JK. (2000) Comparative studies on the suppression of nitric oxide synthase by curcumin and its hydrogenated metabolites through down-regulation of IkappaB kinase and NFkappaB activation in macrophages. *Biochem Pharmacol*, 60:1665-76.

Peng X, Wood CL, Blalock EM, Chen KC, Landfield PW, Stromberg AJ (2003) Statistical implications of pooling RNA samples for microarray Experiments. *BMC Bioinformatics*, Jun 24; 4:26.

Perricone R, Perricone C, Carolis CD, Shoenfeld Y (2008) NK cells in autoimmunity: A two-edg'd weapon of the immune system *Autoimmunity Reviews* 7, 384–390

Peschle C, Rappaport IA, Sasso GF, Gordon AS, Condorelli M. (1972) Mechanism of growth hormone (GH) action on erythropoiesis. *Endocrinology* 91: 511-517.

Peyreigne, C. Bouix, D. Micallef, J.P. Mercier, J. Bringer, J. Prefaut; C. Brun J.F. (1998) Exercise-induced growth hormone secretion and haemorheology during exercise in elite athletes, *Clin Hemorheol Microcirc* 19, 169-176.

Peyreigne, C, Fédou, C, Benhaddad, A A, de Boisvilliers, F, Mercier, J, Bringer, J, Brun, J F (1999) Haemorheology of growth hormone-deficient adults *Clin Hemorheol Micro* 20 167–174.

Pfafferott C, Nash GB, Meiselman HJ (1985) Red blood cell deformability in shear flow. Effects of internal and external phase viscosity and of in vivo aging, *Biophys. J.* 47, 695-704.

Philipps AF, Persson B, Hall K, Lake M, Skottner A, Sanengen T, Sara VR 1988 The effects of biosynthetic insulin-like growth factor supplementation on somatic growth, maturation and erythropoiesis on the neonatal rat. *Pediatr Res* 23 298 –305.

Pichler R, Sfetsos K, Badics B, Gutenbrunner S, Berg J, Auböck J. (2009) Lymphocyte imbalance in vitiligo patients indicated by elevated CD4+/CD8+ T-cell ratio. *Wien Med Wochenschr*;159:337–41.

Polychronakos C, Guyda HJ, Posner BI (1983) Receptors for the insulin-like growth factors on human erythrocytes *J Clin Endocrinol Metab.* Vol. 57, No. 2.

Postel-Vinay MC (1994) Growth hormone receptor and dwarfism. *Revue Practique* 44 1281–1285.

Puniyani RR, Ajmani R, Kale PA. 1991 Risk factors evaluation in some cardiovascular diseases. *J Biomed Eng* 13:441.

Radetti G, Bozzola M, Pagani S, Avanzini, MA, Messner H, Belloni C, Hayakawa M (2000) Circulating GH Isoforms and GH Bioactivity in Preterm Neonates. *Pediatr Res*, 48 (2) 244-247.

Ramel A, Wagner KH, Elmadfa I (2003) Acute impact of submaximal resistance exercise on immunological and hormonal parameters in young men. *J Sports Sci* Dec 21(12):1001-8.

Ramos SB, Brenu E, Christy R, Tajouri L, McNaughton L, Gray B, , Marshall-Gradisnik SM (2010) Assessment of immune function after short-term administration of recombinant human growth hormone in healthy young males. *European Journal of Applied Physiology* 46 1–9.

Rampling MW 1988 Red cell aggregation and yield stress. In: *Clinical Blood Rheology*. Vol. I; GDO, Lowe (Ed.), Boca Raton, FL: CRC Press:45-64.

Riley JK, Takeda K, Akira S, Schreiber RD. (1999) Interleukin-10 receptor signalling through the JAK-STAT pathway: requirement for two distinct receptor-derived signals for anti-infl ammatory action. *J Biol Chem*;274:16513-21.

Rapaport R, Sills IN, Green L, Barrett P, Labus J, Skuza KA, Chartoff A, Goode L, Stene M, Petersen BH (1995) Detection of human growth hormone receptors on IM-9 cells and peripheral blood mononuclear cell subsets by flow cytometry: correlation with growth hormone-binding protein levels. *J Clin Endocrinol Metab.* 80(9):2612-2619.

Redelman D, Welniak LA, Taub D, Murphy WJ (2008) Neuroendocrine hormones such as growth hormone and prolactin are integral members of the immunological cytokine network. *Cell Immunol.* 252:111–121.

Reinhart WH. Fibrinogen--marker or mediator of vascular disease? *Vasc Med*. 2003;8(3):211-6.

Romagnani, S, (1999) Th1/Th2 cells. *Inflamm. Bowel. Dis.* 5, 285–294.

Rosenfeld RG, Attie KM, Frane J, Brasel JA, Burstein S, Cara JF, Chernausek S, Gotlin RW, Kuntze J, Lippe BM, Mahoney CP, Moore WV, Saenger P, Johanson AJ. (1998) Growth hormone therapy of Turner's syndrome: beneficial effect on adult height. J Pediatr. 132(2):319-24.

Saugy M, Robinson N, Saudan, C, Baume N, Avois L, Mangin P 2006 Human growth hormone doping in sport *Br J Sports Med*; 40: 35-39.

Savino W, Postel-Vinay MC, Smaniotto S, Dardenne M (2002) The thymus gland: a target organ for growth hormone. *Scand J Immunol* 55:442–452.

Schottelius AJG, Marty WM, Sartor RB, Baldwin AS (1999) Interleukin-10 Signaling Blocks Inhibitor of κB Kinase Activity and Nuclear Factor κB DNA Binding *The Journal of Biological Chemistry* Vol. 274, 45: 31868–31874.

Seiva, F. R., G. M. Ebaid, et al., (2008). Growth hormone and heart failure: oxidative stress and energetic metabolism in rats. Growth Hormone & IGF Res 18(4): 275-283.

Shalak R, Zarda P, Jan KM, Chien S. (1981) Mechanism of rouleaux formation. *Biophys J* 35:771–781.

Shearer WT, Pahwa S, Read JS, Chen J,Wijayawardana SR, Palumbo P, et al., (2007) CD4/ CD8 T-cell ratio predicts HIV infection in infants: the National Heart, Lung, and Blood Institute P2C2 Study. *J Allergy Clin Immunol*;120:1449–56.

Shimizu K, Adachi K, Teramoto A (2005) Growth hormone enhances natural killer cell activity against glioma. *J Nippon Med Sch*. 72: 335–340.

Shimizu T, Kamegai J, Tamura H, Ishii S, Sugihara H, Oikawa S. (2005) The estrogen receptor (ER) alpha, but not ER beta, gene is expressed in hypothalamic growth hormone-releasing hormone neurons of the adult female rat. *Neurosci Res*, 52(1), 121-125.

Shin, S., Ku, Y., Park, M-S. & Suh, J-S. 2004. Measurement of red cell deformability and whole blood viscosity using laser-diffraction slit rheometer. *Korea-Australia Rheology Journal*, vol.16, no.2, pp. 85-90.

Shin S, Ku Y, Park MS, Suh JS, 2005 Slit-flow ektacytometry: laser diffraction in a rheometer. Cytometry. *B Clin. Cytom*., 65B (1), 6-13.

Shresta S;Pham CT;Thomas DA;Graubert TA;Ley TJ. (1998) How do cytotoxic lymphocytes kill their targets? *Curr Opin Immunol* Oct. Vol.10 (5) 581-7.

Sitz KV, Burks AW, Williams LW, Kemp SF, Steele RW (1990) Confirmation of X-linked hypogammaglobulinemia with isolated growth hormone deficiency as a disease entity. *J Pediatr.* 116(2):292-4.

Smit, LS, Carter-Su C (1996) Growth hormone receptor. Growth Factors and Cytokines in Health and Disease. L. Derek and B. Carolyn, JAI. Volume 1: 43-84.

Sneppen SB, Mersebach H, Ullum H, et al., (2002) Immune function during GH treatment in GH-deficient adults: an 18-month randomized, placebo-controlled, double-blinded trial. *Clin Endocrinol* (Oxf). Dec;57(6):787-92.

Snow EC, Feldbush TL, Oaks JA (1981) The effect of growth hormone and insulin upon MLC responses and the generation of cytotoxic lymphocytes *J. Immunol.* 126: 161-164.

Spencer SD, Di Marco F, Hooley J, Pitts-Meek S, Bauer M, Ryan AM, Sordat B, Gibbs VC, Aguet M. (1998) The orphan receptor CRF2–4 is an essential subunit of the interleukin 10 receptor. *J Exp Med*;187:571-8.

Steffee WP , Goldsmith RS, Pencharz PB, Scrimshaw NS, Young VR (1976) Dietary protein intake and dynamic aspects of whole body nitrogen metabolism in adult humans. *Metab. Clin. Exp.* 25: 281-297.

Stephenson, JR, Lee J M, Bailey N, Shepherd AG, Melling J (1991). Adjuvant effect of human growth hormone with an inactivated flavivirus vaccine. *J Infect Dis*, 164(1), 188-191.

Stoltz JF, Donner M (1987) Haemorheology: Importance of erythrocyte aggregation. *Clin Hemorheol* 7:15.

Tak PP, Firestein GS (2001) NF-kappaB: a key role in inflammatory diseases. *J Clin Invest* 107:7-11.

Tian ZG., Woody MA, Sun R, Welniak LA, Raziuddin A, Funakoshi S, Tsarfaty G, Longo DL, Murphy, WJ (1998) Recombinant human growth hormone promotes hematopoietic reconstitution after syngeneic bone marrow transplantation in mice. *Stem. Cells* 16, 193-199.

Tiong TS, Freed KA, Herington AC. (1989) Identification and tissue distribution of messenger RNA for the growth hormone receptor in the rabbit. *Biochem Biophys Res Commun* 158:141–148.

Tiong, T. S., and A. C. Herington. (1992) Ontogeny of messenger RNA for the rat growth hormone receptor and serum binding protein. *Mol. Cell. Endocrinol*. 83:133–141.

Valerio G, Bond HM, Badolato R, Petrella A, Di Maio S, Salerno M, Waters MJ, Venuta S, Tenore A. (1997) Expression of growth hormone receptor by peripheral blood lymphocytes in children: evaluation in clinical conditions of impaired growth. *Clin Endocrinol* (Oxf). Sep;47(3):329-35.

S Varma, P Sabharwal, J F Sheridan, W B Malarkey (1993) Growth hormone secretion by human peripheral blood mononuclear cells detected by an enzyme-linked immunoplaque assay. *The Journal of Clinical Endocrinology & Metabolism* 76, 149-53

Vaya A, Falco C, Feron O, Contreras T, Walls M. Aznar J (2003) Erythrocyte aggregation determined with the Myrenne aggregometer at two modes (M0, M1) and at two times (5 and 10 sec), *Clin. Hemorheol. Microcirc*. 29, 119–127.

Vayá A, Martínez Triguero M, Ricart A, Plumé G, Solves P, Correla D, Romagnoli M (2009) Erythrocyte aggregability and ABO blood groups. *Clin Hemorheol Microcirc*; 41(1): 67-72.

Veldhuis JD, Johnson ML, Faunt LM, Mercado M, Baumann G (1993) Influence of the High-Affinity Growth Hormone (GH)-Binding Protein on Plasma Profiles of Free and Bound GH and on the Apparent Half-Life of GH Modeling analysis and clinical applications. *J Clin Invest*. 91(2): 629–641.

Wallace JD, Cuneo RC, Baxter R, Orskov H, Keay N, Pentecost C, Dall R, Rosén T, Jørgensen JO, Cittadini A, Longobardi S, Sacca L, Christiansen JS, Bengtsson BA, Sönksen PH (1999) Responses of the growth hormone (GH) and insulin-like growth factor axis to exercise, GH administration, and GH withdrawal in trained adult males: a potential test for GH abuse in sport. *J Clin Endocrinol Metab.* 84(10):3591-601.

Weigent DA, Riley JA, Galin FS, LeBoeuf RD, Blalock JE. 1991 Detection of growth hormone and growth-hormone releasing hormone-related messenger RNA in rat leukocytes by the polymerase chain reaction. *Proc Soc Exp Biol Med*. 198:643– 648

Welle S, Thornton C, Statt M & McHenry B (1996). Growth hormone increases muscle mass and strength but does not rejuvenate myofibrillar protein synthesis in healthy subjects over 60 years old. *J Clin Endocrinol Metab* 81, 3239

Weng X, Cloutier G, Beaulieu R, Roederer GO (1996) Influence of acute-phase proteins on erythrocyte aggregation. *Am J Physiol Heart Circ Physiol* 271:H2346–H2352.

Wenisch C, Patruta S, Daxbock F, Krause R, Horl W (2000) Effect of age on human neutrophil function. *Journal of Leukocyte Biology.* 67(1): 40-45.

Whitney AR, Diehn M, Popper SJ, Alizadeh AA, Boldrick JC, Relman DA, Brown PO. (2003) Individuality and variation in gene expression patterns in human blood. *Proc Natl Acad Sci USA*. 100:1896–1901.

Winer LM, Shaw MA, Baumann G. (1990) Basal plasma growth hormone levels in man: new evidence for rhythmicity of growth hormone secretion. *J Clin Endocrinol Metab.* 70(6):1678-86.

World Anti-Doping Agengy (WADA) (2009) The World Anti-Doping Code The 2009 Prohibited List International Standard, Montreal, Canada http://www.wada-ama.org.

Wu¨hl E, Haffner D, Offner G, Broyer M, van't Hoff W, Mehls O (2001) European Study Group on Growth Hormone Treatment in Children with Nephropathic Cystinosis. Long-term treatment with growth hormone in short children with nephropathic cystinosis. *J Pediatr* 138:880 – 888.

Yarasheski, K.E., Campbell, J.A., Smith, K., Rennie, M.J., Holloszy, J.O., Bier, D.M. (1992). Effect of Growth Hormone and Resistance Exercise on Muscle Growth and Strength in Older Men. *Am J Physiol.* 262(3 Pt 1):E261-7.

Yarasheski KE, Zachweija JJ, Angelopoulos TJ, Bier DM (1993) Short-term growth hormone treatment does not increase muscle protein synthesis in experienced weightlifters. *Journal of Applied Physiology,* 74(6), 3073 - 3076.

Yarasheski, K.E., Campbell, J.A., Khort WM (1997). Effect of resistance exercise and growth on bone density in older men. *Clin Endocrinol* (47), 222-229.

Yasukawa, K, Hirano, T, Watanabe, Y, Muratani, K, Matsuda, T, Nakai, S, Kishimoto, T. (1987) Structure and expression of human B cell stimulatory factor-2 (BSF-2/IL-6) gene. *EMBO J.* 6, 2939–2945.

Zamai L, Mariani AR, Zauli G, Rodella L, Rezzani R, Manzoli FA, Vitale M (1998) Kinetics of *in vitro* natural killer activity against K562 cells as detected by flow cytometry. *Cytometry* 32:280–285.

Zhang W, Carriquiry A, Nettleton D, Dekkers JC (2007) Pooling mRNA in microarray experiments and its effect on power. *Bioinformatics.* May 15;23(10):1217-24. Epub 2007 March 7.

Zhou J, Meadows GG (2003) Alcohol consumption decreases IL-2-induced NF-κB activity in enriched NK cells from C57BL/6 mice. *Toxicol Sci* 73:72-79.

Andiran, N., & Yordam, N. (2007). TNF-[alpha] levels in children with growth hormone deficiency and the effect of long-term growth hormone replacement therapy. *Growth Hormone & IGF Research*, 17(2), 149-153.

Aquino Neto, F. R. d. (2001). O papel do atleta na sociedade e o controle de dopagem no esporte. *Revista Brasileira de Medicina do Esporte*, 7, 138-148.

Auernhammer, C. J., & Strasburger, C. J. (1995). Effects of growth hormone and insulin-like growth factor I on the immune system. *Eur J Endocrinol,* 133(6), 635-645.

Baeza, I., Alvarado, C., Ariznavarreta, C., Castillo, C., Tresguerres, J. A., & De la Fuente, M. (2008). Effect of growth hormone treatment on lymphocyte functions in old male rats. *Neuroimmunomodulation,* 15(4-6), 279-284.

Caligiuri, M. A. (2008). Human natural killer cells. *Blood*, 112(3), 461-469.

Cherwinski, H. M., Schumacher, J. H., Brown, K. D., & Mosmann, T. R. (1987). Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *J Exp Med*, 166(5), 1229-1244.

Crist, D. M., Peake, G. T., Mackinnon, L. T., Sibbitt, W. L., Jr., & Kraner, J. C. (1987). Exogenous growth hormone treatment alters body composition and increases natural killer cell activity in women with impaired endogenous growth hormone secretion. *Metabolism*, 36(12), 1115-1117.

Fu, Y. K., Arkins, S., Fuh, G., Cunningham, B. C., Wells, J. A., Fong, S., et al. (1992). Growth hormone augments superoxide anion secretion of human neutrophils by binding to the prolactin receptor. *J Clin Invest*, 89(2), 451-457.

Gaytan, F., Romero, J. L., Bellido, C., Morales, C., Reymundo, C., & Aguilar, E. (1994). Effects of growth hormone and prolactin on testicular macrophages in long-term hypophysectomized rats. *J Reprod Immunol*, 27(1), 73-84.

Geffner, M. E., Bersch, N., Lippe, B. M., Rosenfeld, R. G., Hintz, R. L., & Golde, D. W. (1990). Growth hormone mediates the growth of T-lymphoblast cell lines via locally generated insulin-like growth factor-I. *J Clin Endocrinol Metab*, 71(2), 464-469.

Kimata, H., & Yoshida, A. (1994). Differential effect of growth hormone and insulin-like growth factor-I, insulin-like growth factor-II, and insulin on Ig production and growth in human plasma cells. *Blood*, 83(6), 1569-1574.

Kooijman, R., Willems, M., De Haas, C. J., Rijkers, G. T., Schuurmans, A. L., Van Buul-Offers, S. C., et al. (1992). Expression of type I insulin-like growth factor receptors on human peripheral blood mononuclear cells. *Endocrinology*, 131(5), 2244-2250.

Marshall-Gradisnik, S. M., Weatherby, R. P., Deakin, G. B., Coutts, R. A., Meir, R. A., Connellan, P. A., et al. (2008). Natural killer cell activity following six weeks of strength training in healthy young males with/without testosterone enanthate administration: ePublications@SCU.

Merchav, S., Tatarsky, I., & Hochberg, Z. (1988). Enhancement of erythropoiesis in vitro by human growth hormone is mediated by insulin-like growth factor I. *Br J Haematol*, 70(3), 267-271.

Mercola, K. E., Cline, M. J., & Golde, D. W. (1981). Growth hormone stimulation of normal and leukemic human T-lymphocyte proliferation in vitro. Blood, 58(2), 337-340.

Moore, K. W., de Waal Malefyt, R., Coffman, R. L., & O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol*, 19, 683-765.

Mosmann, T. R., & Coffman, R. L. (1989). Heterogeneity of cytokine secretion patterns and functions of helper T cells. *Adv Immunol*, 46, 111-147.

Mozaffari, F., Hansson, L., Kiaii, S., Ju, X., Rossmann, E. D., Rabbani, H., et al. (2004). Signalling molecules and cytokine production in T cells of multiple myeloma-increased abnormalities with advancing stage. *Br J Haematol*, 124(3), 315-324.

Sneppen, S. B., Mersebach, H., Ullum, H., & Feldt-Rasmussen, U. (2002). Immune function during GH treatment in GH-deficient adults: an 18-month randomized, placebo-controlled, double-blinded trial. *Clin Endocrinol (Oxf)*, 57(6), 787-792.

Stephenson, J. R., Lee, J. M., Bailey, N., Shepherd, A. G., & Melling, J. (1991). Adjuvant effect of human growth hormone with an inactivated flavivirus vaccine. *J Infect Dis*, 164(1), 188-191.